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14. ABSTRACT

Acinetobacter baumannii is an emerging Gram negative pathogen that has been a major contributor to the significant increases in multi-drug resistant (MDR) nosocomial infections worldwide. This bacterium has been known to rapidly acquire mobile genetic elements, facilitating the emergence of MDR and pan-resistant strains. With the emergence of strains greatly increasing in resistance, treatment with conventional antibiotics has become limited. Furthermore, the development of new antibiotics has not caught up with the spread of antibiotic resistance, and the pharmaceutical antibiotic drug development pipeline is scarce. While antibiotic resistance poses great risk

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DEVELOPMENT AND CHARACTERIZATION OF AN UNMARKED HEMO

CLUSTER KNOCKOUT IN HYPERVIRULENT ACINETOBACTER BAUMANNII

LAC-4

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

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In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in

Biological Sciences

By

Peter James Ewing

March 2016

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March 2016

ABSTRACT

Development and Characterization of an Unmarked *hemO* Cluster Knockout in

Hypervirulent *Acinetobacter baumannii* LAC-4

By

Peter James Ewing

Acinetobacter baumannii is an emerging Gram negative pathogen that has been a
major contributor to the significant increases in multi-drug resistant (MDR) nosocomial
infections worldwide. This bacterium has been known to rapidly acquire mobile genetic
elements, facilitating the emergence of MDR and pan-resistant strains. With the
emergence of strains greatly increasing in resistance, treatment with conventional
antibiotics has become limited. Furthermore, the development of new antibiotics has not
caught up with the spread of antibiotic resistance, and the pharmaceutical antibiotic drug
development pipeline is scarce. While antibiotic resistance poses great risk to human
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In MDR *A. baumanni* for instance, the mechanisms of resistance are well understood, but
the determinants of virulence are poorly understood. Yet scientists are limited in the
molecular tools they can use to study *A. baumannii* due to its resistance to common
antibiotic selective markers. One factor of virulence that has become a growing field of
study in the past two decades is the role of iron metabolism in virulence. Iron is an
important nutrient for all forms of life, and a necessity for growth and replication. While
mammalian hosts evolved an array of iron limiting mechanisms to sequester the iron
from pathogens, the pathogens themselves have evolved strategies to acquire iron for
both colonization and growth. And while the general understanding of bacterial iron

metabolism is progressing, investigations into iron utilization in *A. baumannii* have been limited. Previously, we and our collaborators showed that a strain of *A. baumannii,* LAC-4, isolated from a Los Angeles County hospital outbreak, exhibited hypervirulence in mice and high serum resistance. The strain was found to express a highly efficient heme utilization system, and after treatment with a heme utilization inhibitor, the hypervirulence was attenuated. Bioinformatics analyses indicated that all *A. baumannii* strains contained a ubiquitous heme utilization gene cluster, but only some strains, including LAC-4, possess a second heme utilization gene cluster containing a heme oxygenase (*hemO)* gene. In this study we have generated an unmarked knockout (KO) mutant of LAC-4 in which the *hemO* gene cluster has been deleted. Construction of this mutant utilized an optimized gene replacement system using homologous recombination of a knockout cassette containing upstream and downstream flanking regions to the *hemO* gene cluster cloned into the suicide vector pMo130. MDR LAC-4 had previously been determined susceptible to apramycin, enabling the use of an apramycin resistance gene linked to the end of the KO cassette to provide selection for the single-crossover of the suicide plasmid. The resistance gene was later removed from the chromosome along with the plasmid by a second-crossover event identified by the efficient use of both *sacB* counter selection and the *xylE* reporter gene. The resulting mutant was evaluated against an established mouse model of bacterial pneumonia and was found to be significantly less virulent than the LAC-4 wildtype strain. Overall, our study shows that an unmarked knockout construct design used in conjunction with s*acB* and *xylE* is an effective method for generating easily identifiable unmarked mutants. Furthermore, and of greater

significance in elucidation of heme metabolism, it was shown that the *hemO* cluster

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Introduction

The discovery of antibiotics has been one of the most significant and beneficial

medical discoveries in human history. In the world prior to antibiotics, lifespans averaged

50 years in the first world, and the morbidity and mortality from bacteria and other

infectious agents was left unchecked (Davies, 2010; National Center for Health Statistics,

2011). It was in the early twentieth century that Paul Ehrlich presented the idea of a

"magic bullet," a chemical or substance that would exert their action solely on the

infectious agent (Aminov, 2010). His idea led him to systematically screen compounds at

a large scale for a treatment for syphilis, caused by *Treponema pallidum*. That process

ultimately discovered Salvarsan and furthermore contributed to the basic platform of drug

discovery strategies for years to come (Aminov, 2010; Ehrlich et al., 1910).

Despite of the discovery of a "magic bullet" for syphilis, other antimicrobial

therapeutics eluded researchers. It was only a decade since the First World War when

Alexander Fleming discovered the inhibitory, antibacterial properties of a substance

excreted by the mold *Penicillium notatum* (Flemming, 1929; Aminov, 2010). A decade

later, penicillin was able to enter mass production due to the work of Howard Florey and

Ernest Chain (Chain et al., 1940). It was also around this time that the basis of

Flemming's discovery, the zone of inhibition, was developed into a platform for

antibiotic discovery by Selman Waksman leading into the golden age of antibiotic

discovery, also considered the dawn of the modern antibiotic era (Schatz et al., 1944).

Over the next twenty years (1940s – 1960s) most of our known antibiotics were

discovered (Lewis, 2013). Many of these discoveries made it into clinical use within just

years of discovery and medical professionals had become optimistic of their newly found arsenal of antibiotics (Lewis, 2013). Unfortunately, resistance to some antibiotics began to appear early on – in fact even before the very first patient was treated with penicillin, what appeared to be resistance was noted by researchers and later discovered to be penicillinase activity (Abraham et al., 1940; Davies et al., 2010). While resistance was disturbing, oddly, optimism was not reduced early on as Salvarsan had already been employed for over forty years and yet no resistance to Salvarsan by *T. pallidum* had developed. Even now, years later, no resistance to Salvarsan by *T. pallidum* has been documented, which is a rare and notable exception to the common development of resistance to antimicrobials by microorganisms (Aminov, 2010; Cha et al., 2004). As more antibiotics were introduced, resistance began to show up, often within several years of introduction (Lewis, 2013). While researchers responded with new or synthetically modified versions of existing antibiotics, a similar course of events would follow, with resistant bacterial strains arising within a few years of their original use (Davies et al., 2010).

The rise of resistant strains is in large part a response to the selective pressure of antimicrobial agents. Mechanisms of resistance such as efflux pumps, decreased penetrability of the cell membrane, degradation of antimicrobials, modification of antibiotic targets, and even an increase in production of targeted enzymes or structures, are a few ways bacteria have shown adaptation to antibiotics (van Hoek et al., 2011). In addition, bacteria have been shown adept at the using horizontal gene transfer, acquiring additional metabolic pathways to counter those blocked by therapeutics, using integrons and other mobile elements, and even simply benefiting from random mutations that

confer resistant phenotypes (Davies et al., 2010; van Hoek et al., 2011). Due to the

selective pressure of antimicrobials, bacterial strains acquired resistance endogenously

through mutation and adaption, as well as exogenously through acquisition of foreign

genetic information (van Hoek et al., 2011). An excellent example is penicillin in the late

1950's, which became largely ineffective against bacterium such as *Staphylococcus*

aureus whom penicillin once had high efficacy against (Saga et al., 2009). It was not until

derivatives of penicillin that were made β-lactamase stable, did it appear that penicillin's

failure could be redeemed (Kong et al., 2010). Unfortunately, the very same methods of

acquiring resistance played out numerous times leading to the advent of bacterium that

had acquired multiple resistances, also often called multi-drug resistant (MDR) bacteria

when resistance emerges to at least one antimicrobial agent in at least three or more

categories of antibiotics (Falagas et al., 2006; Nathwani et al., 2014; van Hoek et al.,

2011). In the case of *S. aureus,* the bacterium became resistant to the β-lactamase stable

methicillin in the 1990's (MRSA), and was exacerbated by some strains horizontally

acquiring vancomycin resistance from enterococci as well (VRSA) (Saga et al., 2009;

Kong et al., 2010). And while many strains are still treatable with conventional

antibiotics, the lead the pharmaceutical companies were able to keep ahead of bacterial

resistance has fallen behind in recent years as more and more MDR bacterial strains

began to emerge (Davies et al., 2010). Endogenous and exogenous acquisition of

resistance may still account for much of acquired antibiotic resistance, but resistant strain

emergence has also been accelerated through the over use and often misuse of antibiotics

in medicine as well as agriculture (Capita et al., 2010; Jagusztyn-Krynicka et al., 2008).

Overuse and misuse has in effect artificially selected for those strains harboring

resistance genes and has created the potential danger of MDR strains someday becoming dominant in their respective species.

The rise in prevalence of MDR bacterial strains has dramatically increased in recent years alarming medical professionals and public health officials alike (Boucher et al., 2009). Infections once treatable with penicillin, such as those caused by *S. aureus*, were found to be increasingly resistant, with the clinical prevalence of MRSA jumping from 20-25% in the 1990s, to 50-60% of all *S. aureus* infections in the late 2000s (Association for Professionals in Infection Control and Epidemiology, 2010). Likewise, *Clostridium difficile* infections amounted to 250,000 a year in 2013, causing 14,000 deaths (Centers for Disease Control and Prevention, 2013). In the span of two years, *C. difficile* infections have risen to half a million with 15,000 deaths directly attributed to the pathogen (Centers for Disease Control and Prevention, 2015). And of those infections, recent studies have suggested that upwards of 80% of *C. difficile* strains are resistant to at least one antibiotic, and 27.5% are multidrug resistant (Curry et al., 2009; Tenover et al., 2012). Furthermore, beyond the morbidity and mortality of these MDR infections, the cost alone in medical expenses due to *C. difficile* is over $4.8 billion each year. This is in addition to economic costs from the loss of work experienced by affected patients (Centers for Disease Control and Prevention, 2015).

Overall, a majority of hospital infections are being caused by emerging MDR strains of six pathogens: *Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species (Boucher et al., 2009; Rice, 2008). Also known as the "ESKAPE" pathogens, they have caused significant increases in morbidity and mortality (Boucher et al., 2009; Rice 2008). The

emergence of increasingly MDR and even pan-resistant strains demands new and novel antibiotics for clinical use. While a few new antimicrobials have been developed in recent years, the number of new antimicrobials and antibiotics is still failing to keep pace with the spread of resistance (Bassetti et al., 2013; Boucher et al., 2013). In particular, innovations in the treatment of Gram-negative species have shown little progress with a near absence of any drug candidate active against often MDR Gram-negative bacilli, such as *Enterobacter* species, *P. aeruginosa* and *A. baumannii* (Bassetti et al., 2013; Boucher et al., 2013).

Of particular interest is *A. baumannii*, an opportunistic, aerobic, Gram-negative, non-motile bacillus that is commonly associated with various nosocomial infections, most notably ventilator associated pneumonia (Maragakis et al., 2008; Peleg et al., 2008). Over the past twenty years, its clinical significance has risen by its incredible ability to acquire resistance elements, upregulate existing resistance elements as needed, and various other resistance mechanisms that have given strains near immunity to all antimicrobial classes aside from polymixins, fosfomycin and glycylcyclines (Boucher et al., 2013; Maragakis et al., 2008). While the incidents of community acquired MDR *A. baumannii* infections may be low, the prevalence of hospital acquired MDR infections has been increasing, with 7,300 MDR *A. baumannii* infections in 2013, resulting in 500 deaths (Centers for Disease Control and Prevention, 2013). In addition to ventilator-associated pneumonia, typical *A. baumannii* infections include septicemia, surgical site infections, meningitis, and urinary tract and catheter-associated infections (Gaynes et al., 2005; Maragakis et al., 2008; Ou et al., 2015). Treating these infections can be challenging due to *A. baumannii's* natural resistance to most β-lactams, its efflux pumps,

poor cell permeability, and its acquisition and integration of mobile genetic elements that have increased its repertoire of antibiotic resistance genes (Ou et al., 2015). Regardless of these attributes though, much of what spurs this species' virulence is still unknown (Gaynes et al., 2005; Dijkshoorn et al., 2007). *A. baumannii* as a species is not known to be associated with secreting any host-damaging molecules or other virulence factors that can explain damage to the host's cell, yet the virulence remains (Tomaras et al., 2008; Roca et al., 2012). And while virulence and MDR do not necessarily go hand in hand, the emergence of MDR and pan-resistant strains has been seen as the only plausible factor to fuel *A. baumanii*'s virulence (Antunes et al., 2011). The focus on overcoming antibiotic resistance has driven most *A. baumnannii* research, even in our own lab, to focus on novel antibiotic discovery, either in discovering new novel compounds or identifying new targets to be inhibited (Silva et al., 2014; Tilloston et al., 2013). It is little wonder that research has and continues to focus on new targets as modern antibiotics only target less than 40 of 200 or more essential genes most bacterium possess (Lange et al., 2007; Pucci, 2006).

An alternative approach to combating bacterial pathogens has been emerging. Instead of focusing on antibacterial drugs that kill or inhibit growth of bacterial cells (targeting essential processes), a new paradigm focuses on targeting or hindering virulence factors such as secretory systems, biofilm formation, toxin production, quorum sensing functions and more (Barczak et al., 2009; Duncan et al., 2012; Sintim et al., 2010). The concept of disabling or attenuating a virulence factor has a wide range of ramifications in its potential ability to change the entire course of a disease or infection (Barcazk et al., 2009). One significant aspect of this approach is examining bacterial

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virulence factors that allow bacteria to overcome the natural barriers to infection, such as the host innate immune system and iron sequestration (Runyen-Janecky, 2013; Wandersman et al., 2004). In particular, overcoming iron sequestration and its critical role in various bacterial metabolic processes, makes it absolutely necessary for the survival and growth of the pathogen within the host (Haley et al., 2011; Zimbler et al., 2009). As such, interests in iron uptake systems and metabolism have blossomed in the past decade, even becoming a significant focus in our own lab.

Iron is a critical metabolic requirement for most living systems due to its ability to serve as both an electron donor and an acceptor (Gkouvatsos et al., 2012). Iron's utilitarian nature makes it ideal for physiological and metabolic pathways such as the electron transport chain, serving as a co-factor in nucleotide synthesis, and even in sensing ambient conditions (Lill, 2009). Furthermore, studies have shown that nearly half of all enzymes contain at least one metal ion, with iron being the third most common metal ion used in known characterized enzymes (Andreini et al., 2008; Frawley et al., 2014; Waldron et al., 2009). Understandably then, the ability of microbial pathogens to acquire iron from the host environment is crucial for successful infection and thus it is no surprise that mammalian hosts have evolved iron sequestration systems to limit the amount of free-iron available for pathogens (Haley et al., 2001; Johnson et al., 2012; Wandersman et al., 2004). In recent years, researchers have examined and determined much of how *S. aureus* and *P. aeruginosa* use siderophores and other iron uptake mechanisms to overcome host iron sequestration, yet this line of research in *A. baumannii* has been limited (Gaddy et al., 2012; Mihara et al., 2004). Genomic studies of *A. baumannii* have identified the presence of three different siderophore biosynthesis gene

clusters, ferric (Fe^{3+}) and ferrous (Fe^{2+}) uptake systems, and even a heme uptake system (Antunes et al., 2011; Ejilkelkamp et al., 2011; Mortensen et al., 2013; Zimbler et al., 2009). While siderophore and traditional iron uptake systems have been better characterized, it is heme uptake systems that have only recently begun to draw attention (Antunes et al., 2011). This focus on heme uptake and metabolism is important, because mammalian hosts possess transferrins, iron binding glycoproteins that are able to sequester nearly all free iron in the blood (Gkouvatsos et al., 2012). Thus a bacterial pathogen must overcome this sequestration in the bloodstream, or find an alternative and abundant source of iron in circulation, such as heme, in order to successfully invade and develop a systemic septicemia (Gkouvatsos et al., 2012; Wandersman et al., 2004).

The enhanced ability to utilize heme as a source of iron is a possible explanation for how some *A. baummannii* pathogenic strains successfully acquire iron within the free-iron poor environment of the circulatory system. For example, along with our collaborators, we have previously identified a hypervirulent strain of *A. baumannii*, LAC-4, which possesses an uncanny enhanced ability to acquire heme from its host (de Léséleuca et al., 2014). *A. baumannii* LAC-4 was one of 20 isolates of *A. baumannii* that our lab obtained from Los Angeles County hospital outbreaks in 1997 (Valentine et al., 2008). LAC-4 was discovered to be hypervirulent in mice due to extrapulmonary dissemination that occurred within 24 hours of initial infection (Harris et al., 2013). Furthermore, septicemia and death followed within 48 hours of the initial intranasal inoculation (Harris et al., 2013). Notably, when infected mice were treated with a gallium protoporphyrin IX (GaPPIX) heme uptake inhibitor shortly after inoculation with LAC-4, dissemination out of the lungs and bacteremia did not occur, and the mice survived to

clear the pathogen (de Léséleuca et al., 2014). Our genomic analysis of LAC-4 has identified two separate heme uptake systems present, the one ubiquitous to all *A. baumannii* species, and another, named the *hemO* gene cluster, that is less common but present in 76% of the *A. baumannii* hospital strains we have acquired (Ou et al., 2015). Thus, it is this second gene cluster that is of primary interest to one of our lab's main research focuses: *A. baumannii* heme and iron uptake and metabolism.

The *hemO* gene cluster of LAC-4 consists of eight genes (ABLAC_16780 – ABLAC_16850), many of which are homologous to genes in heme uptake and utilization gene clusters already identified and characterized in *P. aeruginosa* that are implicated in virulence (Fig. 1) (Faure et al., 2013; Hom et al., 2013; Llamas et al., 2009; Ochsner et al., 2000). Of note, this cluster includes the outer membrane receptor gene *hasR* (ABLAC_16800), a *tonB* gene (ABLAC_16830), *VreR* and *VreI* genes (ABLAC_16780 and ABLAC_16790), and lastly a heme oxygenase gene, *hemO* (ABLAC_16840) (Ou et al., 2015). All but the latter of the genes, *hemO*, appear to be involved in a TonB-dependent receptor likely involved in bringing heme across the outer-membrane (Antunes et al., 2011; Llamas et al., 2009). The *hasR* gene encodes the actual outer membrane receptor, whereas the *tonB* gene encodes for the protein TonB, which makes the actual contact with the outer-membrane receptor to initiate active transport across the membrane (Llamas et al., 2009). The *VreR and VreI* genes encode an extra-cyctoplasmic function (ECF) sigma factor along with its corresponding anti-sigma factor respectively (Llamas et al., 2009). Both of these factors form a protein complex with the TonB protein prior to contact with the outer-membrane receptor (Llamas et al., 2009). Lastly, heme oxygenase plays a key role in performing oxidative cleavage on heme, releasing ferrous iron (Fe^{2+})

(Antunes et al., 2011). Additionally, analysis of the *hemO* gene cluster have identified

three ferric uptake regulator (FUR) sequences spaced throughout the cluster that mediate

iron-responsive gene regulation of the cluster (Fuangthong et al., 2003; Ou et al., 2015).

These sequences respond to a FUR protein that acts as a repressor when intracellular

concentrations of ferrous iron reach a threshold level (Fuangthong et al., 2003). This is of

interest, as it suggests a down-regulation of the *hemO* gene cluster transcription when

levels of intracellular iron are adequate for normal cellular function and growth.

Conditions in which the *hemO* gene cluster transcription would not be repressed, would

be those of iron-poor or iron-limited environments, such as the iron-sequestered

environment found in the mammalian cardiovascular system.
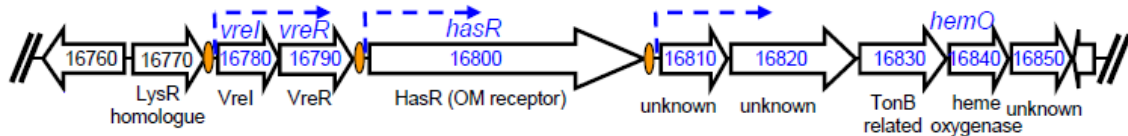
Figure 1. Schematic diagram of the *hemO* gene cluster in *A. baumannii* LAC-4. Genes belonging to the cluster are labeled in blue text. Ferric uptake regulator sequences are indicated by golden ovals between genes. Numbers on each gene refer to the last five digit of LAC-4 locus tags, with "ABLAC_" omitted. Image is modified from schematic by Howard Xu (unpublished).

We hypothesize that the eight-gene *hemO* cluster (HOC) plays an integral role in

A. baumannii LAC-4's hypervirulence, in particular its extreme efficiency in

metabolizing heme, and especially its ability to rapidly undergo extrapulmonary

dissemination and cause extreme septicemia and death. In order to test this hypothesis,

we have employed molecular techniques to create an unmarked *A. baumannii* LAC-4

hemO gene cluster knockout (LAC-4 ΔHOC). A successful knockout was then tested by

our collaborators in an established mouse model for bacterial pneumonia (Harris et al.,

2013). This comparison of the gene-phenotype relationship between wildtype LAC-4 and

LAC-4 ΔHOC has provided elucidation on the role of the HOC in LAC-4's hypervirulence.

One of the primary barriers in advancing research on virulence mechanisms in *A. baumannii,* as well as generating our *hemO* gene cluster knockout, has been a lack of molecular genetic tools that can be used on this increasingly MDR species (Karlowsky et al., 2003; Peleg et al., 2008; Valentine et al., 2008). Selection markers are inevitably limited for MDR clinical isolates of *A. baumanii* as these isolates are resistant to most common antibiotics, rendering most selection markers ineffective in the MDR *A. baumanii* strains. This can be overcome by creating markerless or unmarked knockouts that either do not employ a selection marker in the knockout process, or recycle the "precious" selection marker at the end of the knockout procedures. In order to construct our unmarked *hemO* gene cluster knockout we utilized homologous recombination using the RecBCD system, a system allowing replacement of an existing gene with a knockout construct that contains one or two homologous regions to the targeted gene (Aranda et al., 2010; Dillingham et al., 2008). Homologous recombination can be a one-step or two-step process in which at minimum a single cross-over event is undergone, a second crossover event can follow resulting in either complete recombination or reversion. In order to modify the traditional homologous recombination approaches to create an unmarked mutant, it has been demonstrated that antibiotic selection markers can be placed outside of the knockout construct – as opposed to within the construct, resulting in the selection marker's excision along with the rest of the plasmid during the second-crossover event (Amin et al., 2013; Oh et al., 2015). This methodology required us to rely heavily upon the use of counter-selection in the form of the *sacB* gene to select for cells in which the

second-crossover event occurs, which may create either a knockout mutant or a wildtype revertant (Armin et al., 2013; Pucci, 2006). Already in *A. baumannii*, this has been accomplished by Armin et al., (2013) and Oh et al., (2015) who utilized a *sacB* counterselection to generate knockouts in MDR *A. baumannii* strains 1656-2, DB and R2. The *sacB* gene used by the prior studies lacks in efficiency and repeatability, even though the *sacB* encoded levansucrase cleaves sucrose, producing levans which are toxic to the bacterial cells. Further examination of the literature gives examples of *sacB* counterselection often not working or working inefficiently (Hemlo et al., 2015). Our own lab's prior work with *sacB* counterselection has made us also aware of its shortcomings, and prepared us to optimize it for use.

In response to *sacB*'s inconsistency as a counterselection agent, we optimized the conditions of counter-selection, as well as employed a reporter gene to identify those colonies that have not undergone the second-crossover event. Prior work has shown that media conditions are very particular for sucrose based selection. First, LB medium, which contains salt, is not ideal, instead a basic yeast extract and tryptone medium (YT) is preferred (Amin et al., 2013; Oh et al., 2015). Additionally, the ideal temperatures for the sucrose based selection appears close to 30°C (Blomfield et al., 1991; Oh et al., 2015). Lastly, passaging cells in the YT medium for a day or more has been described for the successful generation of knockouts (Amin et al., 2013). In addition to our optimization of counter-selection conditions, we also have introduced the use of a reporter gene as an integral step to more readily identify potential knockouts and reduce the screening time required in other methods. As a reporter gene we used the *xylE* gene, which is already present on our suicide vector (Hamad et al., 2009). This reporter gene is

useful in that any mutant that still retains the integrated suicide plasmid, thus having gone through only the first crossover event, will display a bright yellow pigment when exposed to catechol (Hamad et al., 2009; Lee et al., 1996). Since the generation of unmarked mutants nearly always requires screening for mutants via colony PCR, this reporter gene has reduced the overall time spent on screening, increasing the rate at which successful knockout mutants were identified.

Due to our lab's focus on iron uptake and metabolism in *A. baumannii*, this unmarked knockout project has been of great benefit in elucidating our understanding of the role of the *hemO* gene cluster in LAC-4. The *hemO* gene cluster had been detected at a high rate in our hospital acquired strains and had appeared likely to contribute to increased virulence, possibly even LAC-4's hypervirulence (Oh et al., 2015) Here we present how we constructed an unmarked *hemO* gene knockout to test its contribution to virulence. This unmarked knockout was constructed using an optimized gene knockout protocol utilizing the RecBCD mediated homologous recombination system in coordination with *sacB* counter-selection and the *xylE* reporter gene. After verification and characterization of our *hemO* gene cluster knockout, we were able to comparatively test the mutant's virulence *in vivo* with the help of our collaborator, Dr. Chen. The resulting virulence assay has increased our understanding of the *hemO* gene cluster, and allowed us to present data suggesting its contribution LAC-4's hypervirulence. This study furthermore helps elucidate a greater understanding of heme metabolism in *A. baumannii* and may contribute to developing anti-virulence strategies in *A. baumannii* infections in the future.

CHAPTER 2

Materials and Methods

Bacterial Strains, Plasmids, and Growth Media

The bacterial strain *Acinetobacter baumannii* LAC-4 was obtained from an

undisclosed Los Angeles County Hospital during an *A. baumannii* outbreak in 1997

(Valentine et al., 2008). The suicide vector pMo130 harbored in *E. coli* JM109 was

acquired from addgene (Fig. 2) (Hamad et al., 2009). The recombinant plasmid pMo130

containing the knockout construct was constructed in a cloning host, *Escherichia coli* K-

12 DH5α (Life Technologies, Carlsbad, CA). The pHN1896 plasmid, a gift from Dr.

Nakashima of the National Institute of Advanced Industrial Sciences and Technology

(Sapporo, Japan), was harbored in *E. coli* DH5α cells and used as template for the

apramycin gene (Fig. 3).
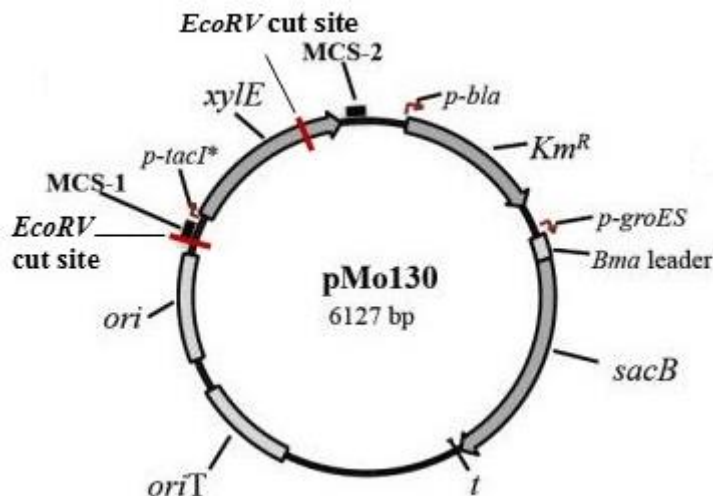
Figure 2. Diagram of the pMo130 suicide vector. This plasmid contains a kanamycin resistance marker (Km^R), *sacB* gene for sucrose based counter selection, and the *xylE* reporter gene. *EcoRV* restriction enzyme cut sites are shown in red; the *SmaI* restriction enzyme cut site is within MCS-1. This image was modified from Figure 1 of Hammand et al., 2010.
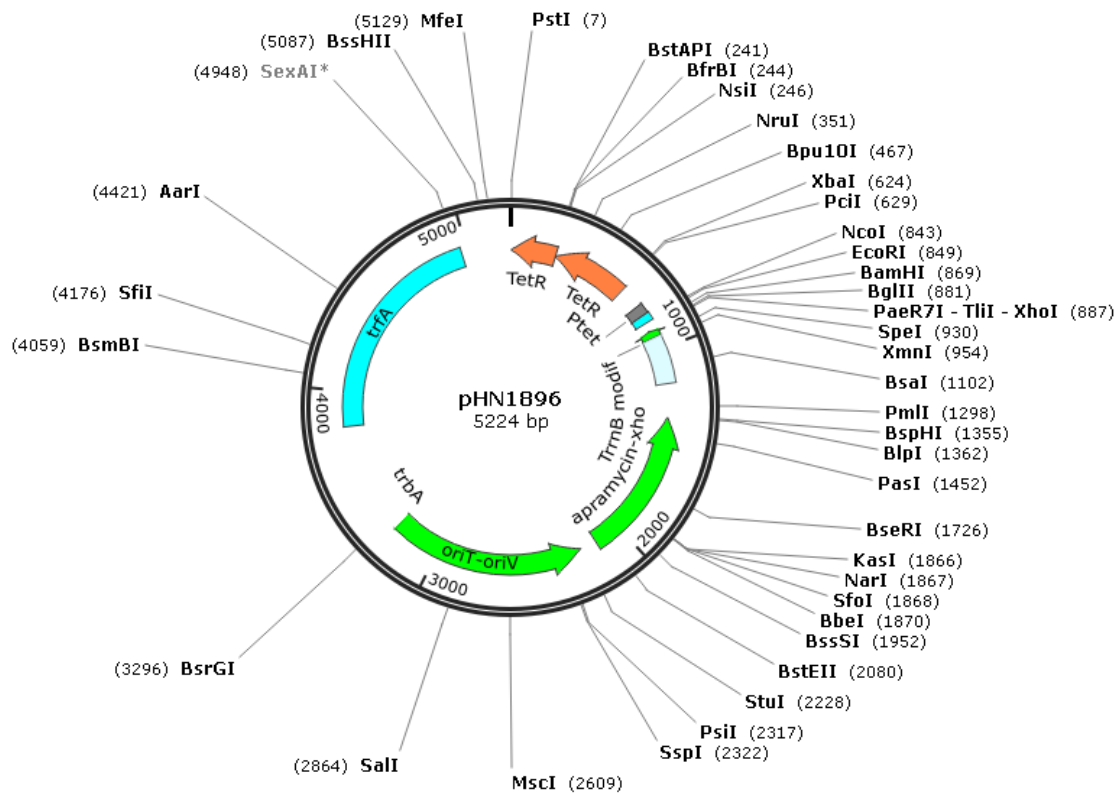
Figure 3. Diagram of the pHN1896 plasmid that contains the apramycin gene. Schematic based upon sequence provided by Dr. Nakashima, 2013.

     A. baumannii and *E. coli* DH5α bacterial cells were grown in Difco Luria Bertani

(LB) medium (Fisher Scientific, Waltham, MA) at 37 °C. *A. baumannii* LAC-4 cells with

integrated knockout construct and suicide vector were counter-selected against utilizing

LB media without salt (YT), made from 5 g Tryptone (Fischer Scientific) and 2.5 g of

Yeast Extract (Fischer Scientific) into 500 ml of Milli-Q purified water. *E. coli* DH5α

harboring the pMo130 plasmid was maintained in LB media supplemented with 30 µg/ml

kanamycin. Agar medium was prepared with the addition of 1.5% Bacto agar (Fisher

Scientific) to LB or YT broth medium as appropriate. For transformations, heat shocked

and electroporated cells were grown in Super Optimal broth with Catabolite repression

(SOC) medium (Invitrogen by Life Technologies). For antibiotic selection, LB medium

augmented with 15 µg/ml of apramycin and 15 µg/ml of kanamycin was utilized to select

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E. coli DH5α containing the recombinant pMo130 plasmid. Single-cross over transformants of *A. baumannii* LAC-4 were selected using LB media with 50 μg/ml apramycin. Counter-selection favoring the double-crossover event utilized YT media supplemented with 15% sucrose in broth, and 10% sucrose in agar medium.

Genomic and Plasmid DNA extraction

Genomic DNA (gDNA) was extracted utilizing the Promega Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Genomic DNA template was diluted to 100 ng/μl for use as template in Polymerase Chain Reaction (PCR).

Large quantities of plasmid vectors were isolated and purified utilizing a Promega Wizard Plus Midipreps DNA Purification System. Recombinant plasmids containing the knockout construct were isolated from liquid culture using the QIAprep Spin Miniprep kit by QIAGEN (Valencia, CA). Any plasmid DNA used as PCR template was first diluted to 100 ng/μl for use in PCR.

Primer Design, Construct Construction, and Overlap Extension PCR

All primers were designed with melting temperatures of approximately 60°C ±2°C. Primers were designed to amplify regions of 400 – 600 base pairs (bp) upstream and downstream of the knockout target for use in homologous recombination with the targeted bacterial genome (Table 1). These amplified upstream and downstream regions were designed to be fused together and then fused with an apramycin gene at the downstream end (Fig. 4). Fusion of the amplified regions, also referred to as fragments, was accomplished by designing the upstream reverse primer to contain an 18 bp tail that matched the first 18 bp of the downstream forward primer. And likewise, the downstream forward primer was designed with an 18 bp tail that matched the first 18 bp of the

upstream reverse primer. Lastly, the downstream reverse primer was designed with a 20

bp tail to match the first 20 bp of the forward primer of an apramycin gene. These

complimentary primer tails would overlap and would allow the upstream and

downstream fragments to be fused together during overlap extension PCR.
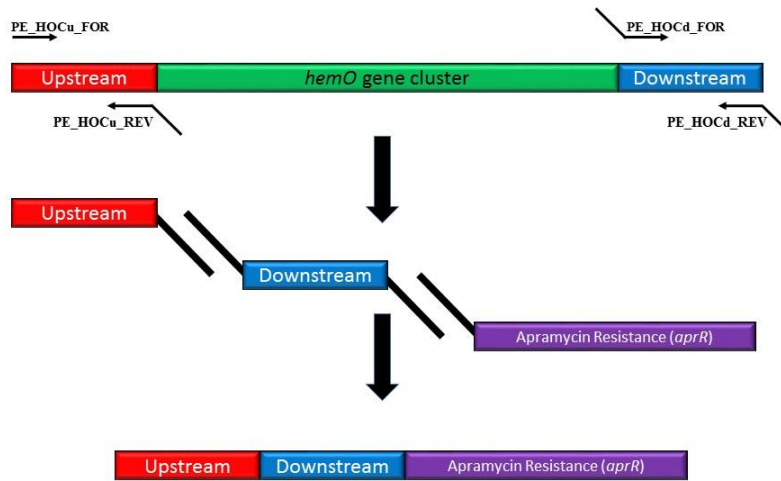
Figure 4. Construction of gene knockout cassette. Upstream and downstream flanking
regions were amplified by PCR. The upstream reverse primer (PE_HOCu_REV)
contained an 18 bp tail matching 18 bp of the downstream forward primer. Similar tails
were placed on both downstream primers (PE_HOCd_FOR and PE_HOCd_REV), with
exception that PE_HOCd_REV contained a 20 bp tail linking to an apramycin cassette.
Constrcut was then stitched together using an overlap extension PCR.

Table 1. *PCR Primers used to construct the hemO gene cluster KO construct.*[a].

Primer Name	Sequence (5' – 3')
PE_HOCu_FOR	GGCAAGGCGTGCATTTAG
PE_HOCu_REV	CAAAAATGCCAGTCATAACTTAAAGCAAATCAGCCAAACTC
PE_HOCd_FOR	TGGCTGATTTGCTTTAAGTTATGACTGGCATTTTTGCATG
PE_HOCd_REV	AGCCCTGGTTAAAAACAAGGCAACACTGTCTTCAAAACAGCAAC
APR_FOR	CCTTGTTTTTAACCAGGGCTG

APR_REV	CCTCCAACGTCATCTCGTTCTC

[a] Underlined portions of sequence indicate overlapping tails.

The apramycin gene and the upstream and downstream flanking regions were amplified via PCR using the following recipe: 2.5 µl forward primer (10 µM), 2.5 µl reverse primer (10 µM), 1 µl LAC-4 gDNA template or pHN1896 for the apramycin fragment (100 ng/µl), 25 µl PfuTurbo Hotstart 2X Master Mix from Agilent Technologies (Santa Clara, CA), and 19 µl of UltraPure Distilled RNase/DNase free water (Invitrogen by Life Technologies) for a total reaction volume of 50 µl. The PCR for individual KO fragment generation used the following parameters: 1 cycle of 95°C for 2 minutes, 30 cycles with a denaturation step at 95°C for thirty seconds, a primer annealing step at 53°C for 30 seconds, and an elongation step at 72°C for 2 minutes, ending in a final elongation step of 72°C for 10 minutes. Amplified fragment generation was verified by agarose gel electrophoresis. The amplified fragments were then purified using the QIAquick PCR Purification Kit (QIAGEN) and concentrated in a final volume of 32 µl of Qiagen elution buffer (EB) and the concentration was determined using the Thermo Fischer Nanodrop ND-1000 Spectrophotometer (Thermo Fisher).

Fusing individual fragments together was done utilizing overlap extension PCR by the following recipe: 1750 fmol of the upstream fragment, 1750 fmol of the downstream fragment, 1750 fmol of the apramycin fragment, 25 µl PfuTurbo Hotstart 2X Master Mix, with the remaining volume being brought up to 50 µl with UltraPure Distilled DNase/RNase free water. Overlap extension PCR took place in two PCR steps modified from an openwetware.org protocol and contained: a set of "Overlap PCR" cycles followed by a set of "Purification PCR" cycles (Vanlang et al., 2011). The "Overlap PCR" cycles had the following parameter: 1 cycle of 95°C for 2 minutes, 15

cycles with a denaturation step at 95°C for thirty seconds, a cycle at 70°C for 30 seconds, followed by a cycle at 72°C for 2 minutes, ending in a cycle of 72°C for 10 minutes. This cycle serves the purpose of priming the templates (Vanlang et al, 2011). After the "Overlap PCR" cycles, 2.5 μl of both forward and reverse primer were added to the reaction and the "Purification PCR" cycles began using the following parameters: 1 cycle of 95°C for 2 minutes, 30 cycles with a denaturation step at 95°C for thirty seconds, a primer annealing step at 53°C for 30 seconds, and an elongation step at 72°C for 2 minutes and thirty seconds, ending in a final elongation step of 72°C for 10 minutes. This final cycle was referred to as "Purification cycle" since only the entire desired construct should be amplified. Successful fusion of the fragments into the KO construct was verified by gel electrophoresis to visualize the presence of the expected band size. The resulting product was purified and concentration was determined as described above.

Ligation of Knockout Construct into pMo130

The pMo130 plasmid vector was linearized using *SmaI* (New England Biolabs, Ipswich, MA). The PCR amplified knockout construct cassette was then blunt end ligated into the linearized pMo130 plasmid using T4 DNA ligase by Life Technologies (Carlsbad, CA). Ligations were performed in two ratios, a 1:2 and a 1:5 ratio of plasmid to insert. Each reaction used 4 μL of T4 DNA ligase buffer, 40 fmol of plasmid, 80 or 200 fmol of insert depending on the above mentioned ratio, 1 μL of T4 DNA Ligase, brought to a total 20 μL reaction volume with UltraPure Distilled DNase/RNase free water. Reactions were incubated overnight for 14-16 hours at 14°C.

Transformation into *E. coli* DH5α & Isolation of Plasmid DNA

Ligation mixtures were transformed into *E. coli* DH5α cells using the standard heat shock transformation protocol provided by Life Technologies. After the heat shock procedure, cells were plated onto LB Agar plates containing 15 µg/ml apramycin and 15 µg/ml kanamycin. Plates were then grown over night at 37°C for 16-18 hours.

Transformants were spot plated onto LB Agar containing 15 µg/ml apramycin and 15 µg/ml kanamycin while simultaneously being inoculated into 10 ml of LB containing 15 µg/ml apramycin and 15 µg/ml kanamycin. Both spot plates and liquid cultures were incubated at 37°C for 16-18 hours, with the latter being in a shaking incubator. Spot plates verified growth on the selective media, while liquid culture was pelleted down for plasmid isolation utilizing the QIAprep Spin Miniprep kit by QIAGEN.

Verification of Recombinant Plasmid

Verification of successful ligation was determined by restriction digest. A restriction digest with *EcoRV* (New England BioLabs), cuts upstream and downstream of the *SmaI* cassette insert site contained in the first multiple cloning site (MCS) (Fig. 2). Restriction digest was prepared with the following recipe for both recombinant and wildtype plasmid: 7 µL of plasmid, 2 µL of NEB Buffer 3.1, 1 µL of *EcoRV* and 11 µL of UltraPure Distilled DNase/RNase free water bringing the reaction to a total volume of 20 µL. Digests were incubated overnight at 37°C followed by imaging via gel electrophoresis.

Additionally, presence of the complete KO construct was verified by PCR using the PE_HOCu_FOR and APR_REV end primers. PCR was performed using the

"Purification" cycle of the overlap extension PCR protocol described above. PCR products were imaged via gel electrophoresis and compared to expected construct size.

Preparation of *A. baumannii* LAC-4 Electrocompetent Cells

Electrompetent *A. baumannii* LAC-4 cells were made as previously described with some modifications (Philips, 2010). One LAC-4 colony was inoculated into 50 ml of LB and grown in a shaking incubator at 37°C. Culture was grown to an OD_{600} of 0.2 to 0.5, at which time 20 ml of the culture was transferred to 500 ml of LB. The new 500 ml culture was placed back into the shaking incubator at 37°C and grown to an OD_{600} of 0.5 to 0.7. Cells were harvested and centrifuged in 500 ml Nalgene Centrifuge Bottles (Fisher Scientific) at 10,000 X g for 20 minutes at 4°C. The supernatant was removed and pellets were wash and re-suspended with 125 ml of chilled 10% glycerol. The wash and resuspension was repeated three times before re-suspending the final pellet in equal volume of chilled 10% glycerol. Aliquots of 100 µl and 200 µl of competent cells were placed in 1.5 ml microcentrifuge tubes (Corning Incorporated, Corning, NY) and flash-frozen for fifteen minutes in an ethanol-dry ice slurry. Electrocompetent cells were stored at -80°C until use.

Transformation of Recombinant Plasmids into *A. baumannii* LAC-4

Recombinant plasmids were electroporated into *A. baumannii* LAC-4 competent cells. Competent cells were thawed out on ice and aliquoted into 50 µl aliquots. 100 ng of recombinant plasmid was added to each aliquot appropriately and incubated on ice for 5 minutes. Contents were transferred to pre-chilled 1 mm pre-sterilized electroporation cuvettes (Molecular BioProducts) and then electroporated on settings A9R in a BioRad MicroPulser Electroporator (Hercules, CA). Following electroporation, 450 µl of SOC

Medium (Invitrogen by Life Technologies) and incubated for an hour at 37°C.

Elecroporated cells were plated onto LB medium containing 50 μg/ml of apramycin and

grown overnight to select for single crossover mutants.

Screening for Single-Crossover Transformants

Successful transformants would have had to integrate the apramycin resistance

gene harboring plasmid into their chromosome via a RecBCD mediated single-crossover

event, as pMo130 is a suicide vector in the *Acinetobacter* species (Fig. 5) (Dillinghamg et

al, 2008). These co-integrants (mutants that have integrated the plasmid and knockout

cassette into their chromosome) would also express the reporter gene, *xylE*. In the

presence of catechol or para-catechol, the reporter gene cleaves the catechol based

substrate and produces 2-hydroxymunoic semialdehyde yielding a bright yellow coloring

in the medium (Lee et al., 1996). *Acinetobacter* is capable of producing its own catechol-

like substrate, and thus no additional exposure to catechol was required (Xu lab

unpublished data).

Figure 5. Co-integration of the plasmid via a Single-Crossover Event. A single-crossover event occurs between the homologous recombination of either the upstream flanking regions or the downstream flanking regions. Upstream recombination is shown above illustrating how the plasmid inserts itself behind the chromosomal upstream flanking region, linking the constructs upstream flanking region to the *hemO* gene cluster in its place.

Colonies on the transformation plates that appeared bright yellow, indicating co-integration of the plasmid and subsequent XylE expression, were simultaneously spot platted onto LB agar containing 50 μg/ml of apramycin and inoculated into 5 ml of LB containing 50 μg/ml of apramycin. Media was incubated overnight at 37°C, with liquid medium incubating in a shaker. Portions of co-integrant colonies were then taken from the spot plate and lysed by inoculating into 300 μl of UltraPure Distilled DNase/RNase free water. Inoculum was then heated to 95°C for 15 minutes followed by centrifuging at 13,000 rpm for 2 minutes. Supernatant was transferred to a new microcentrifuge tube and used for colony PCR to confirm integration of the plasmid.

Colony PCR was performed using the same PCR parameters described above for fragment construction, differing only in the master mix recipe. The master mix used the following recipe: 20 μl of 5 Prime HotMaster Mix (VWR, Radnor, PA), 2.5 μl of forward primer, 2.5 μl of reverse primer, 1 μl of 100 ng/μl template and 24 μl of UltraPure Distilled DNase/RNase free water. Colony PCR utilized two sets of primers: PE_HOC_CFM_UP & ACICU_873-Rev and PE_HOC_CFM_DN & ACICU_879-For.

Passaging and Plating to Select for Double-Crossover Mutants

Single-Cross over mutants verified by both colony PCR and a positive indication of integration of the *xylE* reporter gene were subjected to passaging to apply stress and select for the loss of the *sacB* containing pMo130 plasmid due to a second cross-over event. The verified single-crossover mutants were grown in LB containing 50 μl of apramycin overnight. A 10 μl aliquot of overnight culture was then inoculated into 5 ml of YT broth containing 15% sucrose. The culture was grown at 30°C with 10 μl being passaged to fresh YT broth with 15% sucrose after 24 hours. After three passages the

final culture was measured at OD_{600} using a Beckman Coulter DU-800 Spectrophotometer (Brea, CA) to determine approximate colony forming units (CFU). The culture was then serially diluted and approximately 10^2 cfu were plated onto YT agar containing 10% Sucrose. Plates were then incubated overnight at 30°C.

Replica Plating

Presence of white pigmented colonies, as opposed to yellow XylE expressing colonies, after overnight growth was indication of double-crossover KO mutants or a reversion to wildtype (Fig. 6). Due to the separation of colonies from plating at 10^2 cfu, we discovered that the naturally produced catechol like substance may not be concentrated enough in the media to produce the yellow pigmentation (Xu lab unpublished data). To further enhance the screen, the plates from the passaged mutants were replica plated onto Whatman 185 mm pore filter paper (GE Healthcare Life Sciences, Pittsburg, PA) and subsequently sprayed with 0.5 M catechol (Sigma-Aldrich, St. Louis, MO). Filters were then air-dried overnight while the original plates were grown back at ambient temperature overnight. Colonies that were still harboring the pMo130 plasmid and KO construct would appear yellow on the filter paper. Potential KO's and wildtype reverts though appeared as a dark opaque spot on the filter paper. Filters were then compared to the original plates to identify white colonies which were then spot plated on both LB and LB with 50 µl of apramycin. Colonies that grew only on the LB spot plate but failed to grown on the apramycin containing media were then inoculated into 5 ml of LB for glycerol stock and potential genomic extraction. A portion of the colony remaining was used to prepare colony PCR template as described above.
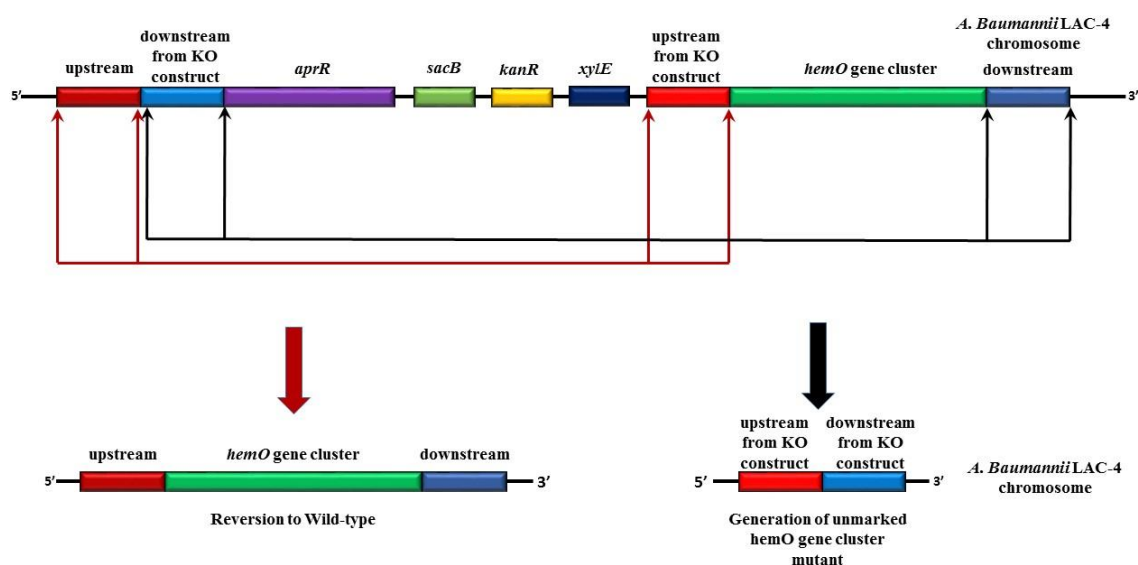
Figure 6. Double-Crossover resulting in Gene Knockout or Wild-type Reversion. After a successful co-integration from a single-crossover event, the remaining flanking region (illustrated here as downstream; black lines show downstream regions pairing) may undergo homologous recombination excising the plasmid backbone and target gene resulting in a gene knockout. Likewise, the original flanking regions that underwent the single-crossover event can recombine again (illustrated here as upstream; red lines show upstream regions pairing) resulting in a reversion to wildtype.

Confirmation Primer Design

Confirmation PCR Primers were designed to amplify several regions of interest to examine the site of deletion as well as confirm the absence of the eight gene *hemO* cluster (Table 2). The site of deletion was examined by designing a forward primer (PE_HOC_CFM_UP) several hundred base pair upstream of the PE_HOCu_FOR primer and a reverse primer (PE_HOC_CFM_REV) several hundred base pairs downstream of PE_HOCd_REV. Additionally, two existing primers within the site of deletion (ACICU_873-Rev and ACICU_879-For) were utilized in conjunction with the above confirmation primers to both identify single-crossover events as well as further confirm a KO mutant (Fig. 7). Additionally, existing primers to screen for genetically conserved

regions within each gene of the *hemO* gene cluster were used to confirm the absence of

the cluster's genes (Fig. 7) (Ou et al., 2015). Lastly, four sets of primers were designed to

verify the presence or absence of genes unique to the pMo130 suicide vector (Fig. 8).

Table 2. *Confirmation PCR Primers used to verify single-crossover and KO mutants.*[b]

Primer Name	Sequence (5' – 3')
PE_HOC_CFM_UP	CGCGGTCGAACTCGG
PE_HOC_CFM_DN	GGATCTCGACATATTACCCACG
ACICU_873-For	GGCTTCACCAATGGTTGAGAC
ACICU_873-Rev	GGTAATGCAAAATAAAAGCTTGCTG
ACICU_874-For	TGGATTGTTCAACTGAGTTCGG
ACICU_874-Rev	CATTGACCCGCACATGTTG
ACICU_875-For	GTGATAACTCTCATTTTTCGGGC
ACICU_875-Rev	AAGGCCACTGCGGAACA
ACICU_876-For	TGCTTGTTCAACTGCATTTGC
ACICU_876-Rev	CTGGGCAATACCCGCTAAAG
ACICU_877-For	ATATGCCGACGAAGACACACAG
ACICU_877-Rev	GGCATTTGCACAACAAAGGTAT
ACICU_878-For	GTTGCCTTTACCACAAGGGC
ACICU_878-Rev	GATTGAGTTTGAGTTGGACTGGAA
ACICU_879-For	AAACAAGAAACTGCTGCTGAACA
ACICU_879-Rev	CTGCCCGTTGGGTTGAA
ACICU_880-For	ATGTCATGGTAAAACATGAGCAGTC
ACICU_880-Rev	TTTAAGCCACATCCACGCC

PE_CFM_KanR_FOR	CAACAGACAATCGGCTGCTC
PE_CFM_KanR_REV	CCACCATGATATTCGGCAA
PE_CFM_SacB_FOR	CAACTCAAGCGTTTGCGAA
PE_CFM_SacB_REV	GTTTGCTAACTCAGCCGTGC
PE_CFM_xylE_FOR	CATGTGCAGCTGCGTGTAC
PE_CFM_xylE_REV	CAGATAGAAACCGAGCACCTTG
PE_CFM_pMo_INT_FOR	CATCCTCTCTCGTTTCATCGG
PE_CFM_pMo_INT_REV	TGTTACGCCGGCGGTAG

[b]*hemO* gene cluster screen primer names derived from their homologous gene locus tags in *A. baumannii* ACICU. All other primers were named using a combination of the designer's initials, gene name, "CFM" to indicate its use as a confirmation primer, and FOR or REV for directionality of each primer in a primer pair.
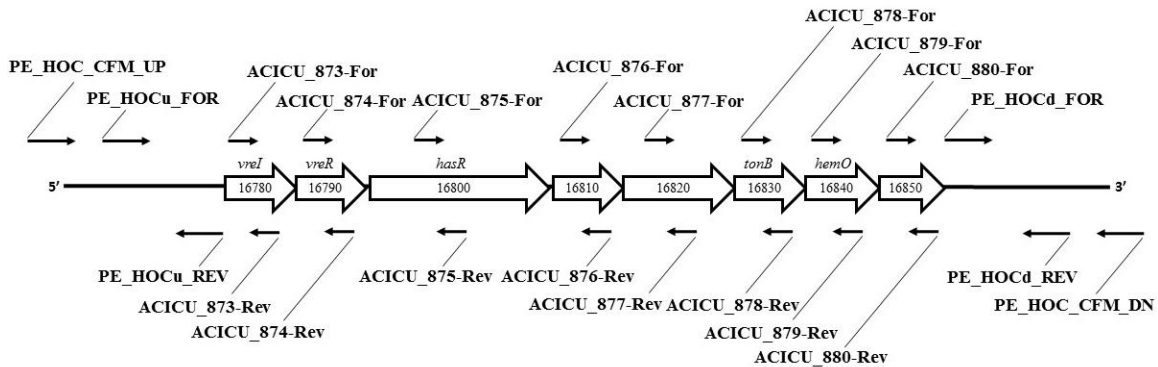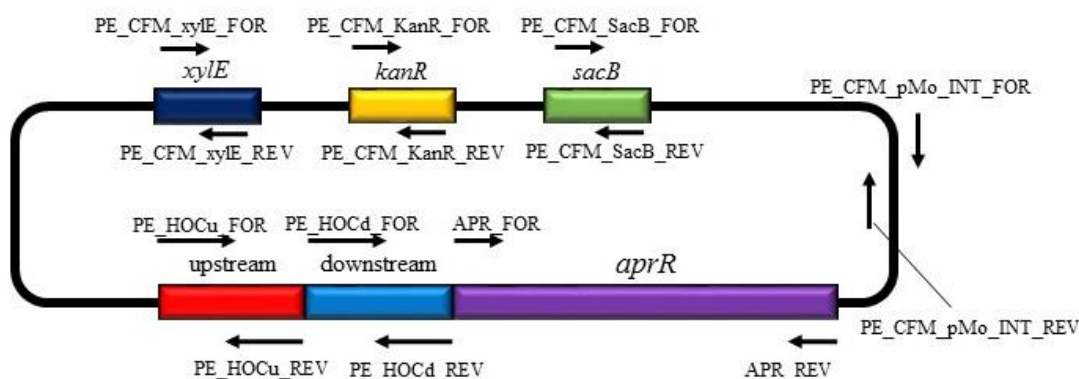

Figure 7. Relative locations of Confirmation Primers for the *hemO* gene cluster. Locations of primers are representative to illustrate approximate locations on the chromosome and are not to scale.

Figure 8. Relative locations of pMo130 backbone confirmation primers. Approximate locations of confirmation primers used to confirm the removal of the pMo130 plasmid after a double-crossover event. Illustration above uses the pMo130 vector with ligated knockout construct and is not to scale.

Confirmation of Cluster Deletion via Colony PCR and genomic DNA PCR

Colony PCR confirmations were designed to quickly screen white colonies for potential KOs. The colony PCR consisted of three primer sets: PE_HOC_CFM_UP & PE_HOC_CFM_DN, PE_HOC_CFM_UP & ACICU_873-Rev, and PE_HOC_CFM_DN & ACICU_879-For. PCR reactions were carried out as described when confirming a single-crossover via colony PCR.

Colonies with white pigmentation that had positive colony PCR results for being a KO were subsequently subjected to genomic extraction after glycerol stocks were made for archiving. Genomic extraction was carried out as described above. Using the same PCR reaction recipe and parameters as described with the colony PCR reactions, sixteen PCR reactions were performed to verify and confirm KO status. The following reactions were used to verify the deletion: PE_HOC_CFM_UP & PE_HOC_CFM_DN, PE_HOC_CFM_UP & ACICU_873-Rev, and PE_HOC_CFM_DN & ACICU_879-For. The excision from the chromosome of pMo130 was confirmed using four backbone primer sets: PE_CFM_KanR_FOR & PE_CFM_KanR_REV, PE_CFM_SacB_FOR&

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PE_CFM_SacB_REV, PE_CFM_xylE_FOR & PE_CFM_xylE_REV, and

PE_CFM_pMo_INT_FOR & PE_CFM_pMo_INT_REV. Lastly, the *hemO* gene cluster

primers (all eight primer sets beginning with "ACICU") were used as further

confirmation that the individual genes of the cluster had in fact been removed with the

cluster.

Sequencing to Verify Site of Deletion

The PCR product from the genomic DNA confirmation of PE_HOC_CFM_UP &

PE_HOC_CFM_DN for confirmed ΔHOC mutant strains was purified using QIAquick

PCR Purification Kit (QIAGEN). Purified product was nanodropped and diluted to 8.3

ng/μl to provide 100 ng in a 12 μl Sanger sequencing reaction. Reactions were pre-mixed

with 20 pmoles of the appropriate primer for both the forward and the reverse reaction.

Sequencing was performed by Retrogen, Inc. (San Diego, CA).

Phenotyping *hemO* Gene Cluster Mutant

Basic phenotyping of the LAC-4 ΔHOC was performed on LB Agar using a disc

diffusion assay. Liquid culture was streaked on the plate in order to create a lawn. Discs

were then placed on the medium and the 10 μl of the following antibiotics were added to

the discs: ampicillin (10 mg/ml), apramycin (10 mg/ml), kanamycin (10 mg/ml),

meropenem (10 mg/ml), tetracycline (12.5 mg/ml) and ciprofloxacin (2.56 ng/ml). Plates

were incubated overnight and zones of inhibition were compared.

In Vivo Murine Studies

In vivo assays of *A. baumannii*'s virulence was carried out by our collaborator Dr.

Wangxue Chen and his group at the National Research Council Canada using an

established murine model for bacterial pneumonia as previously described (Harris et al.,

2013). Mice were inoculated with approximately 2 x 10^7 CFU of fresh culture prepared from frozen stocks of *A. baumannii* wildtype and mutant strains (Harris et al., 2013). Mice were monitored and scored by appearance as per the protocol, and groups composed of three to six mice were sacrificed at 3 time points post-inoculation: 0, 4 and 24 hours (Harris et al., 2013). Lungs, spleen and lymph nodes were removed for bacterial quantification or for histopathological examination (Harris et al., 2013).

CHAPTER 3

Results

Construction of Knockout Cassette and Ligation into Suicide Vector

To knockout the *hemO* gene cluster in *A. baumannii* LAC-4, we began

constructing a knockout cassette containing both flanking regions of the gene cluster

fused together. These flanking regions would be homologous to the same regions in

LAC-4 and could be swapped with the existing regions via the RecBCD mediated

homologous recombination pathway (Dillingham et al., 2008). We began by amplifying

the flanking regions and the apramycin gene fragment that would be linked to the

downstream region and used as a temporary selection marker to select for co-integrants

(Fig. 9). Overlap extension was performed to fuse the gene fragments together into the

hemO gene cluster knockout cassette shown as the expected band size of 1,964 base pair

(Fig. 10). The linearized pMo130 suicide vector was then blunt-end ligated with the
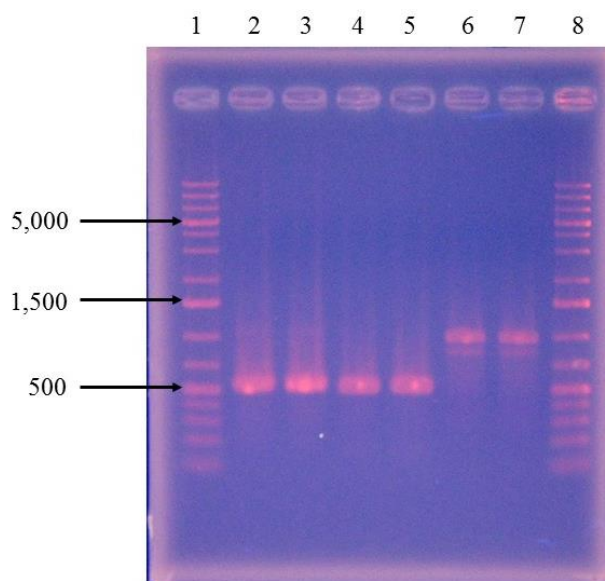
knockout construct.

Figure 9. Confirmation of successful gene fragment amplification of apramycin and
hemO gene cluster upstream and downstream flanking regions. PCR samples were run on

1% agarose gel in comparison to GeneRuler 1kb DNA Ladder. DNA bands were visualized using ethidium brome (EtBr) and UV light. Lane 1 & 8: GeneRuler 1kb DNA Ladder; lane 2 & 3: HOC upstream flanking region (531 bp); lane 4 &5: HOC downstream flanking region (505 bp); lane 6 & 7: *apr* gene enconding apramycin.
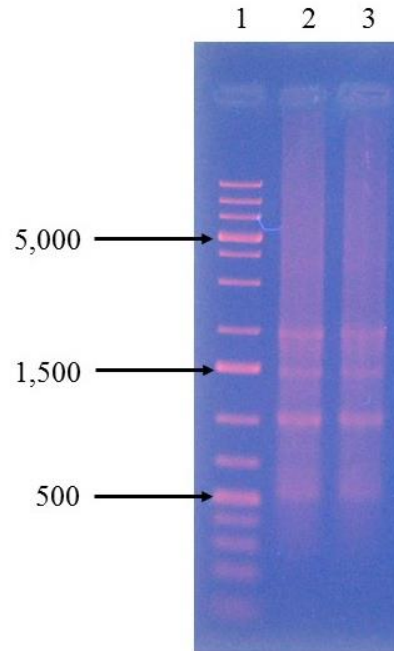

Figure 10. Confirmation of successful overlap extension of the *hemO* gene cluster KO cassette. PCR samples were run on 1% agarose gel in comparison to GeneRuler 1kb DNA Ladder. DNA bands were visualized using EtBr and UV light. Lane 1: GeneRuler 1kb DNA Ladder; lane 2 & 3: *hemO* gene cluster KO cassette (1964 bp).

Transformation into *E. coli* DH5α and Isolation of Recombinant Plasmid

Ligation mixtures were transformed into *E. coli* DH5α competent cells via heat-shock since pMo130's pMB1 origin of replication is recognizable by *E. coli*. The presence of growing colonies is an indication of successful transformation.

Recombinant plasmid was isolated from transformants via plasmid miniprep. The recombinant plasmid was characterized by restriction digest with *EcoRV*, a two-cutter that cuts around the MCS, and compared to a digest of wildtype pMo130 (Fig. 11). The resulting digest of the recombinant plasmid matched the calculated sizes for two bands: 5,304 bp and 2,787 bp compared to 5,304 bp and 823 bp respectively for pMo130 without the knockout cassette. The recombinant plasmid's smaller 2,787 bp band, which

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contains the MCS, was 1,964 bp larger than corresponding smaller band on non-recombinant pMo130, thus matching the knockout constructs size and indicating its presence on that band (Fig. 2). Additional PCR verification was performed on the recombinant plasmid using the upstream forward primer and the apramycin reverse primer to confirm a complete construct was present.
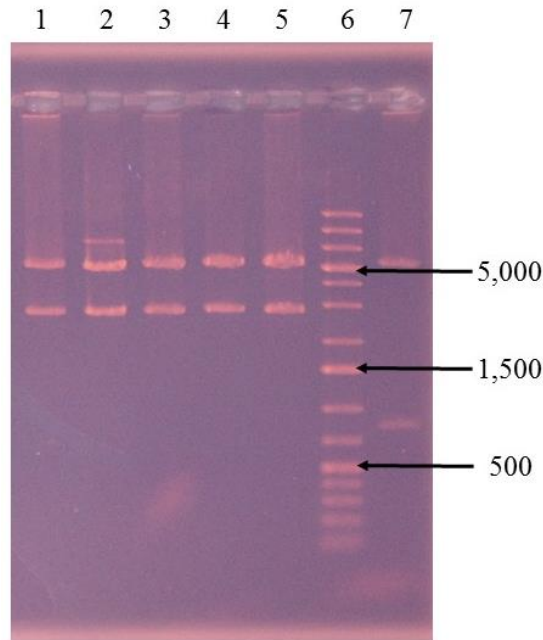
Figure 11. EcoRV restriction digest of recombinant plasmid miniprep. Digests were run on 1% agarose gel in comparison to GeneRuler 1kb DNA Ladder. DNA bands were visualized using EtBr and UV light. Lane 1 through 5: pMo130 with HOC KO cassette insert (5,304 & 2,787 bp); lane 6: GeneRuler 1kb DNA Ladder; lane 7: pMo130 with no insert (5,304 & 823 bp).

Transformation into electrocompetent *A. baumannii* LAC-4

To introduce the recombinant plasmid into *A. baumannii* for RecBCD mediated integration of the suicide plasmid through a single-crossover event, the recombinant plasmid was electroporated into electrocompetent *A. baumannii* LAC-4 cells (Fig. 12) (Dillingham et al., 2008). Robust colonies were selected and spot plated against a grid on LB agar supplemented with 50 μg/ml of apramycin.
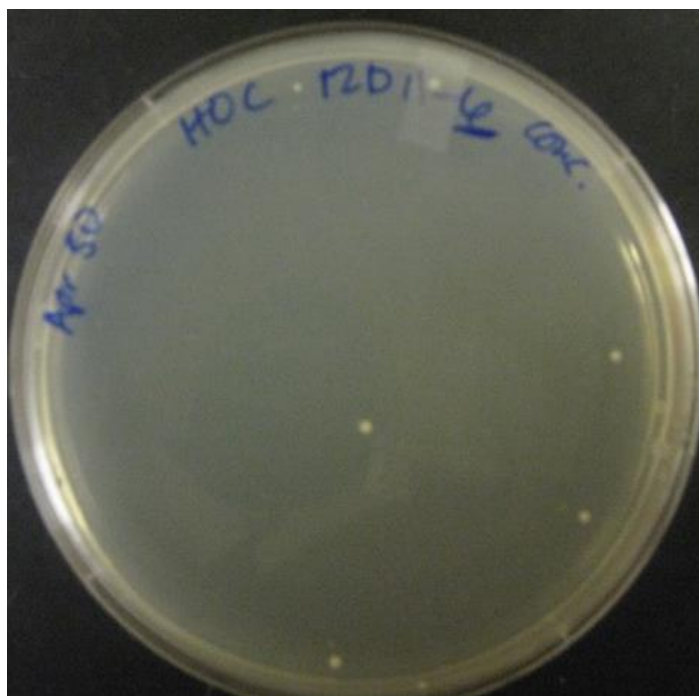
Figure 12. Transformation of pMo130 with KO cassette into electrocompetent *A. baumannii* LAC-4 cells. Transformed cultures were plated onto LB agar supplemented with 50 μg/ml of apramycin.

Verification of Single-Crossover Mutant

Overnight incubation revealed spot plates containing white pigmented colonies as well as bright yellow pigmented colonies, in which the yellow pigment appeared to seep into the medium surrounding the expressing colonies (Fig. 13). This result was unexpected, but the cause was readily apparent for what had occurred. The pMo130 plasmid contains the reporter gene *xylE* encoding for a catechol-2,3-dioxygenase, which acts upon colorless catechol-like substrates and converts them into bright yellow 2-hydroxymunoic semialdehyde compounds (Hamad et al., 2009; Lee et al., 1996). The results suggest that *A. baumannii* LAC-4 produces a catechol-like substance that *xylE* can act upon. This fortuitous event allowed us readily identify single-crossover mutants without replica plating and spraying with catechol. Similarly, the liquid culture that had been inoculated at the time of spot platting also exhibited bright yellow pigmentation for

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the same colonies that shown as such on the spot plates (Fig. 14). As an additional step, we chose to four quadrant streak from the yellow pigmented overnight cultures onto LB agar supplemented with 50 μg/ml of apramycin to isolate single colonies for colony PCR verification (Fig. 15).
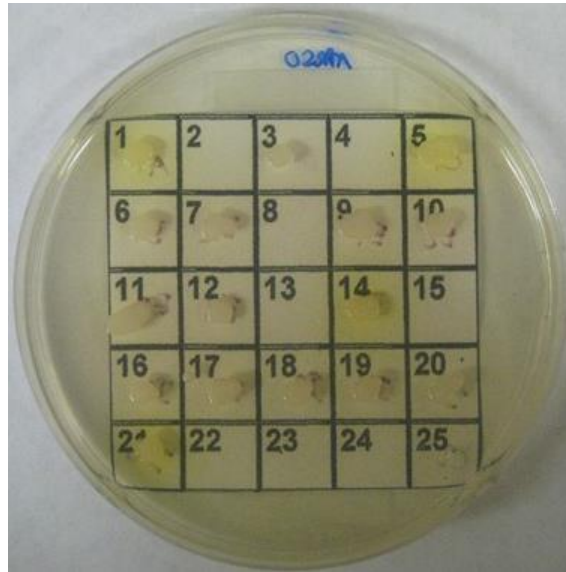
Figure 13. Spot plate of transformant *A. baumannii* LAC-4 colonies. Transformants were spotted onto LB agar supplemented with 50 μg/ml apramycin. Successful transformants expressing the *xylE* gene appear yellow in color; potentially false positives appear white in color.
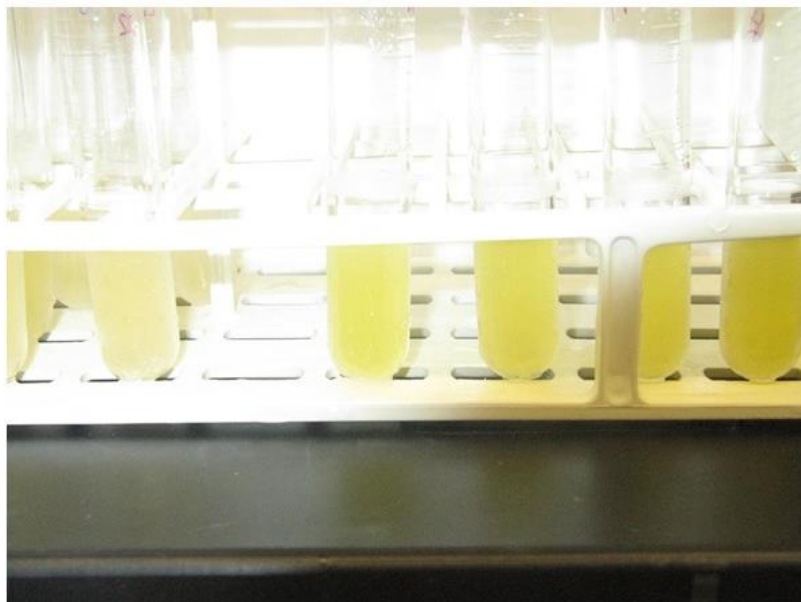
Figure 14. Overnight liquid culture of transformant *A. baumannii* LAC-4 colonies. Transformants were inoculated into 5 ml of LB supplemented with 50 μg/ml apramycin.

Successful transformants (four tubes on right) are expressing *xylE* and show a bright yellow color; false positives (tubes on left) exhibit a dull yellow color.

Figure 15. Single-crossover HOC transformant four quadrant streaked for isolation of single colonies. Transformant was streaked onto LB agar supplemented with 50 μg/ml apramycin. Note the bright yellow pigmentation due to expression of the *xylE* reporter gene.

To confirm the single-cross over event, two colony PCR reactions were performed from an isolated colony from the putative co-integrant mutant. The primer set PE_HOC_CFM_UP & ACICU_873-Rev was used to analyze a genomic region that begins internal to the site of deletion and goes beyond the entire upstream flanking region, generating a 1,389 bp band if the upstream site was not disrupted by the single-cross over event. Likewise, the primer set PE_HOC_CFM_DN & ACICU_879-For was used to detect a genomic region that begins internal to the site of deletion and goes beyond the entire downstream flanking region, generating a 1,672 bp band for an undisrupted downstream flanking region. The resulting bands showed an intact

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downstream site and no band for the upstream site, suggesting the upstream site was

disrupted during the insertion of the plasmid during the single-crossover event (Fig. 16).
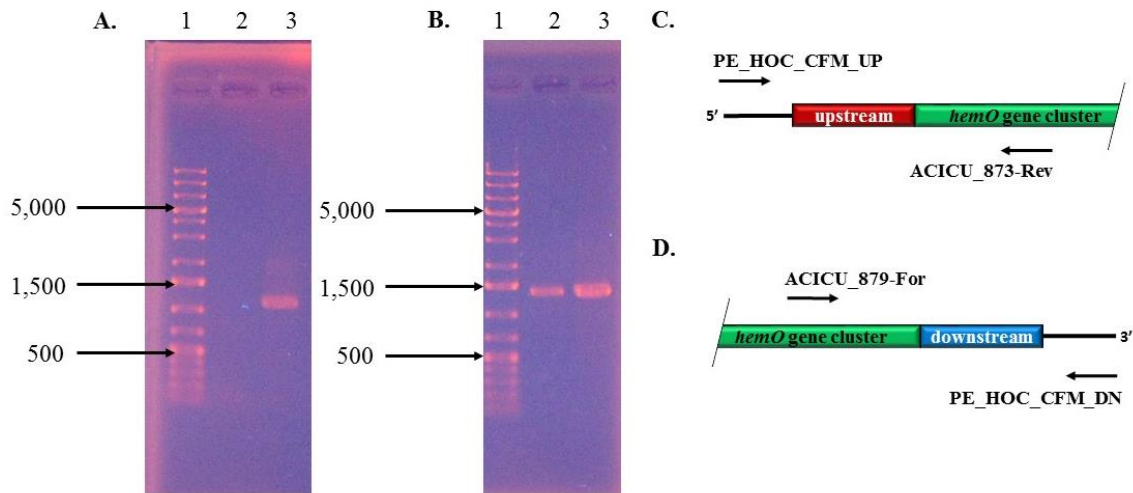

Figure 16. Colony PCR confirmation of single-crossover transformant. PCR samples were run on 1% agarose gel in comparison to GeneRuler 1kb DNA Ladder. DNA bands were visualized using EtBr and UV light. **A**. Analysis of upstream flanking region for single-recombinant event. Lane 1: GeneRuler 1kb DNA Ladder; lane 2: LAC-4 HOC transformant, lane 3: LAC-4 (1,389 bp). **B**. Analysis of downstream flanking region: Lane 1: GeneRuler 1kb DNA Ladder; lane 2: LAC-4 HOC transformant (1,672 bp); lane 3: LAC-4 (1,672 bp). **C.** Diagram of confirmation primer orientation around the upstream site for homologous combination corresponding to upstream gel shown in A. **D.** Diagram of confirmation primer orientation around the downstream site for homologous combination corresponding to upstream gel shown in B.

Passaging and Plating for Second-Crossover Mutants

A single colony from the confirmed single-cross over plate was passaged for

three days before being plated on YT medium. After 16-18 hours of incubation, the plates

had colonies generally well separated from each other (Fig. 17). Some colonies could

visually be seen to be yellow pigmented, others appeared to be white in pigmentation.

During optimization of this protocol, it was determined that denser groups of colonies

containing single-cross over mutants were quicker to change to a bright yellow color, as

opposed to a single colony distant from other colonies. Thus white pigmented colonies,

given enough time could possibly turn bright yellow – this is likely due to the amount of

catechol-like substrate available for *xylE* in the extracellular medium. To expedite our ability to identify double-crossover mutants or wildtype revertants, we replica plated the plates and sprayed the replica filters with catechol. Plates were left on the bench for the colonies to grow back at ambient temperatures as the filters dried. After at least half a day to an entire day of air drying, the filters showed opaque colonies on the filters where no *xylE* activity was seen (Fig. 18). The *xylE* negative colonies were then identified on the original YT with 10% sucrose plate and spot platted on LB agar and LB agar containing 50 μg/ml apramycin simultaneously. Plates were incubated overnight at 37°C. White colored colonies that grew on LB agar but failed to grow on LB agar supplemented with 50 μg/ml apramycin were identified as potential second-crossover KO mutants or wildtype revertants (Fig 19).
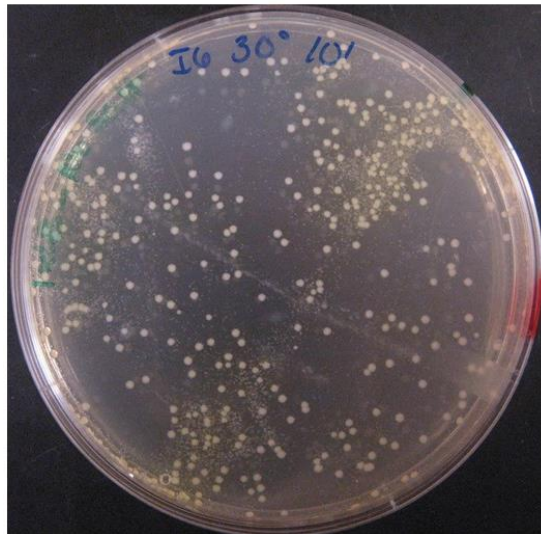
Figure 17. Plating transformants for counter-selection after three days of passaging. Above if an example of HOC transformants that were passaged for three days in YT media supplemented with 15% sucrose before finally being plated onto YT agar supplemented with 10% sucrose for counter selection.
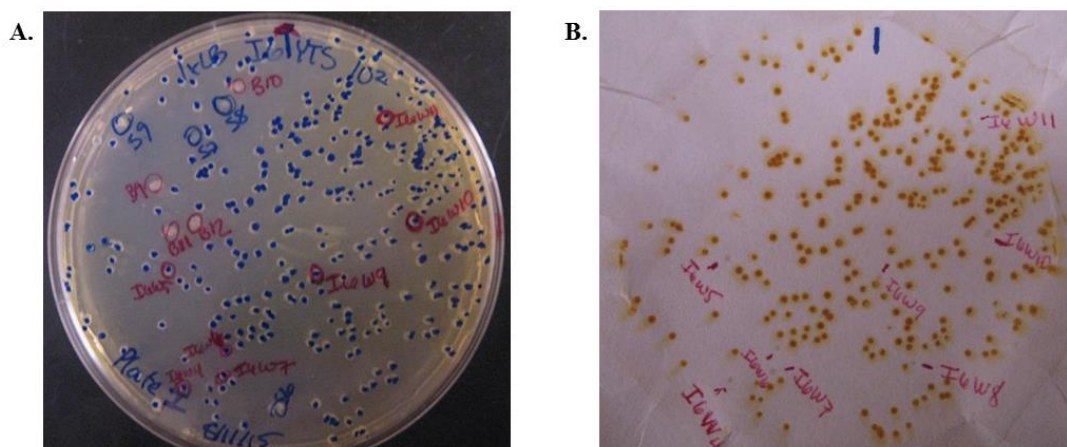
Figure 18. Replica plating and identification of potential knockout mutants on the original plate. **A.** Original plate marked to more easily view colonies. White pigmented colonies circled and labeled. **B.** Replica filter after 24 hours. After replica filters had been given time to adequately dry, opaque or white pigmented colonies were identified on the filter and the located on the original plate.
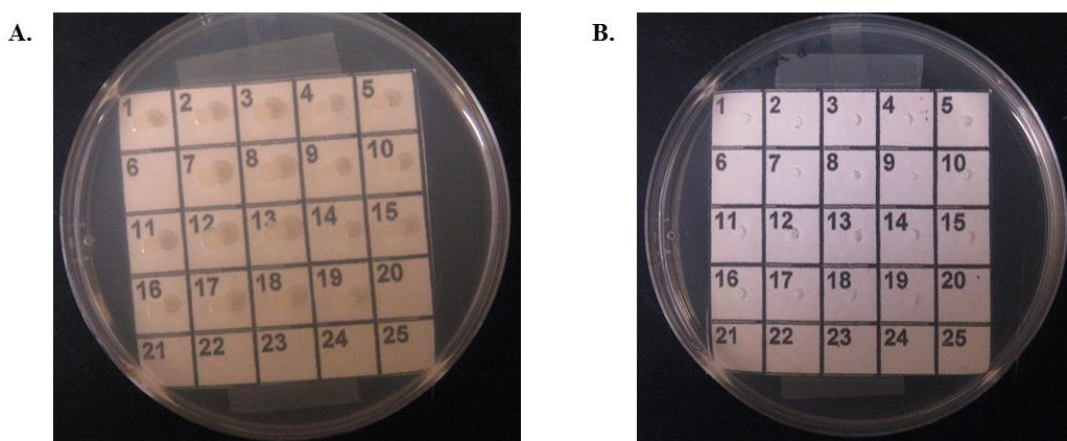
Figure 19. Spot plating white pigmented colonies on both LB medium and selective medium to confirm absence of the selection marker. **A.** LB agar medium. **B.** LB agar supplemented with 50 μg/ml apramycin. White pigmented colonies from the replicated plates were spotted onto both plates depicted above. Absence of growth on the corresponding apramycin plate indicated absence of the resistant selection marker.

Confirming Deletion of the *hemO* Gene Cluster

Potential mutants or wildtype revertants underwent Colony PCR against three

primer sets: PE_HOC_CFM_UP & PE_HOC_CFM_DN, PE_HOC_CFM_UP &

ACICU_873-Rev, and PE_HOC_CFM_DN & ACICU_879-For. The primer set

PE_HOC_CFM_UP & PE_HOC_CFM_DN was the primary confirmation primer set as

it covers the entire site of deletion generating 1,515 bp band in a successful deletion and a

11,090 bp band in wildtype (which cannot be generated and appears as no band given the

PCR reaction conditions) (Fig. 20). The second and third primer sets were the two sets

used to confirm that a single-crossover had occurred. They were used here to confirm

both the knockout through the absence of their expected band sizes (due to ACICU_873-

Rev and ACICU_879-For primers both binding within the site of deletion) and that the

isolated knockout is a pure colony and not mixed with single crossovers. To reduce the

number of colony PCR reactions performed, we instead modified our protocol to only run

those samples showing the 1,515 bp positive KO band for PE_HOC_CFM_UP &

PE_HOC_CFM_DN, and then only those positive bands against ACICU_879-For &

ACICU_879-Rev to confirm absence of an internal region to the *hemO* gene, 240 bp (Fig.

21).
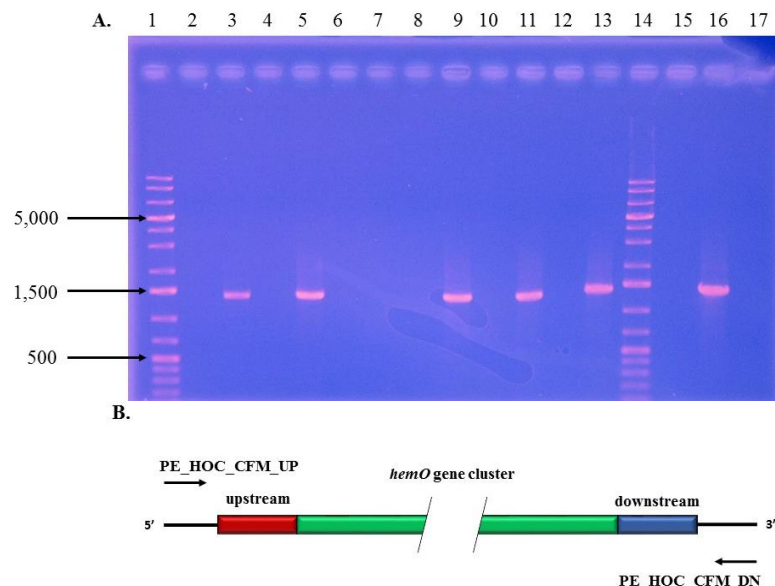
Figure 20. Colony PCR confirmation of mutants covering the site of deletion. PCR
samples were run on 1% agarose gel in comparison to GeneRuler 1kb DNA Ladder.

DNA bands were visualized using EtBr and UV light. **A.** Lane 1 & 14: GeneRuler 1kb DNA Ladder; lane 2-13: potential LAC-4 ΔHOC transformants (1,515 bp); lane 15: pMo130 plasmid with KO cassette; lane 16: LAC-4 HOC single-crossover; lane 17: LAC-4 wildtype. **B.** Diagram of confirmation primers around the entire of deletion which would generate a 1,515 bp band in a knockout, but no band in wildtype due to the size of the *hemO* gene cluster.
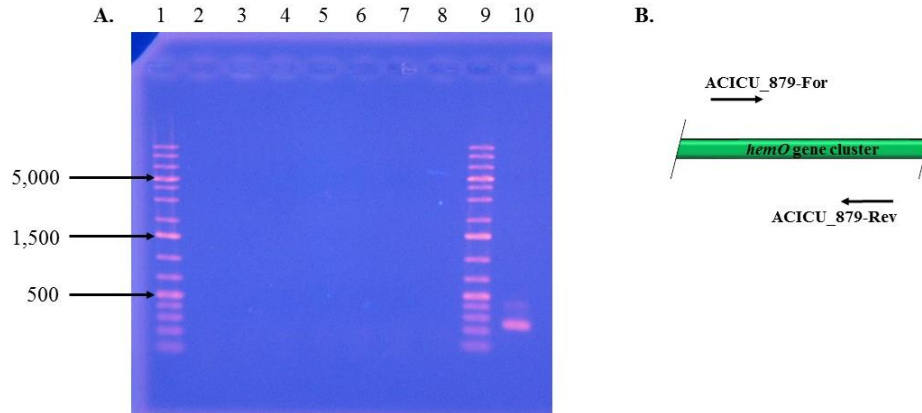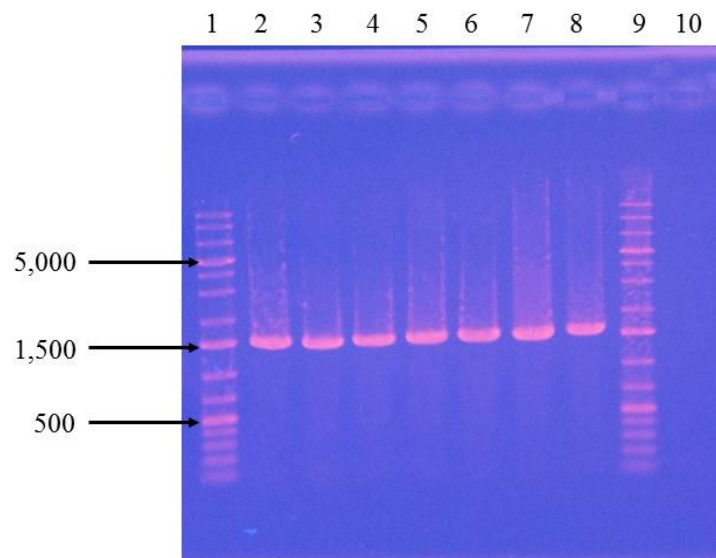

Figure 21. Colony PCR confirmation of mutants for presence of the *hemO* gene. PCR samples were run on 1% agarose gel in comparison to GeneRuler 1kb DNA Ladder. DNA bands were visualized using EtBr and UV light. **A.** Lane 1 & 9: GeneRuler 1kb DNA Ladder; lane 2-8: potential LAC-4 ΔHOC transformants; lane 10: LAC-4 wildtype (240 bp). **B.** Diagram of internal confirmation primers within the *hemO* gene cluster used to verify the removal of a conserved region within the *hemO* gene.

Colonies that colony PCR verified as putative *hemO* cluster KO mutants underwent genomic extraction, and the genomic DNA was used in multiple PCR confirmations to verify deletion and absence of all genes within the *hemO* gene cluster. Even though some of these reactions had just been completed using colony PCR, the use of higher quality genomic DNA template was preferred. Furthermore, from prior optimization in the lab it has been noted that absence of a positive result on colony PCR may be due to poor template quality and thus a false negative result was obtained. In the event of confirming a gene knockout that relies upon a negative result to confirm deletion, it is imperative to use the highest quality template possible.

PCR on gDNA was initially completed for PE_HOC_CFM_UP & PE_HOC_CFM_DN, PE_HOC_CFM_UP & ACICU_873-Rev, and ACICU-879-For &

ACICU-879-Rev primer sets, and once again deviated from our initial protocol by leaving out the PE_HOC_CFM_DN & ACICU_879-For due to its mistaken absence in the single-crossover mutant. The choice to remove confirmation PCR of PE_HOC_CFM_DN & ACICU_879-For was an error only realized after all colony PCRs, genomic PCRs and sequencing had already been done, as it was actually the upstream flanking region that had been disrupted and not the downstream end. This test still would identify wildtype revertants, but would give false-negatives on co-integrants that still harbored the plasmid. It should have been the PE_HOC_CFM_UP & ACICU_873-Rev primer set that was removed if any. Ultimately, due to the robust PCR analysis performed, errors were avoided, and KOs were accurately identified. The PE_HOC_CFM_UP & PE_HOC_CFM_DN primer set generated a 1,515 band as expected for all seven putative *hemO* gene cluster knockouts, and as expected no band was generated for LAC-4 wildtype (Fig. 22). The PE_HOC_CFM_UP & ACICU_873-Rev primer set generated no bands for any of the seven putative knockouts, but still generated a 1,389 bp band on LAC-4 wildtype as expected given ACICU_873-Rev binds within the site of deletion (Fig. 23). Lastly, ACICU-879-For & ACICU-879-Rev generated a 240 bp band in LAC-4 wildtype representing an internal region within the *hemO* gene, while no bands were generated for the putative knockouts (Fig. 24). Additionally, the putative knockouts were screened against four other primer sets to verify the complete removal of pMo130's plasmid backbone. The primer set PE_CFM_KanR_FOR & PE_CFM_KanR_REV screened for a 520 bp region internal to pMo130's KanR gene (Fig. 25). Presence in the LAC-4 ΔHOC-4 mutant appears likely, and possibly in LAC-4 ΔHOC-2 and LAC-4 ΔHOC-9, though it is possibly non-specific

binding to a similar kanamycin resistance gene in *A. baumannii* LAC-4 (Ou et al., 2015).

The primer set PE_CFM_SacB_FOR & PE_CFM_SacB_REV was used to screen for an

860 bp band internal to pMo130's SacB gene (Fig. 26). Presence may be indicated in

LAC-4 ΔHOC-7, LAC-4 ΔHOC-8 and LAC-4 ΔHOC-9 mutants. The primer set

PE_CFM_XylE_FOR & PE_CFM_XylE_REV screened for a 501 bp region internal to

the *xylE* gene (Fig. 27). Presence of the *xylE* gene may be shown in all samples except for

LAC-4 ΔHOC-1 and LAC-4 ΔHOC-2. This result is very likely due to some non-specific

binding of a similar region within *A. baumannii* LAC-4, whether functional or not, as

faint binding can be seen with LAC-4 wildtype on the gel. This same banding pattern has

occurred on subsequent gene knockouts performed in the Xu lab regardless of other

screens being positive for the knockout (Xu lab unpublished data). The final backbone

primer set, PE_CFM_pMo_INT_FOR and PE_CFM_pMo_INT_REV generated a 630 bp

band representative of an intergenic region within the pMo130 plasmid (Fig. 28). No

bands were seen for any of the putative knockouts. In order to determine complete

removal of the *hemO* gene cluster a final screen for conserved regions within all eight

genes of the *hemO* cluster was performed as described before (Fig. 29) (Ou et al., 2015).

Absence of the *hemO* cluster genes was indication of successful deletion of the cluster.

Figure 22. Genomic DNA PCR confirmation of mutants covering the site of deletion. PCR samples were run on 1% agarose gel in comparison to GeneRuler 1kb DNA Ladder. DNA bands were visualized using EtBr and UV light. Lane 1 & 9: GeneRuler 1kb DNA Ladder; lane 2-8: potential LAC-4 ΔHOC transformants (1,515 bp); lane 10: LAC-4 wildtype. See Fig. 21B for diagram of the PCR primers used in this assay. See Fig. 20B for primer orientation.
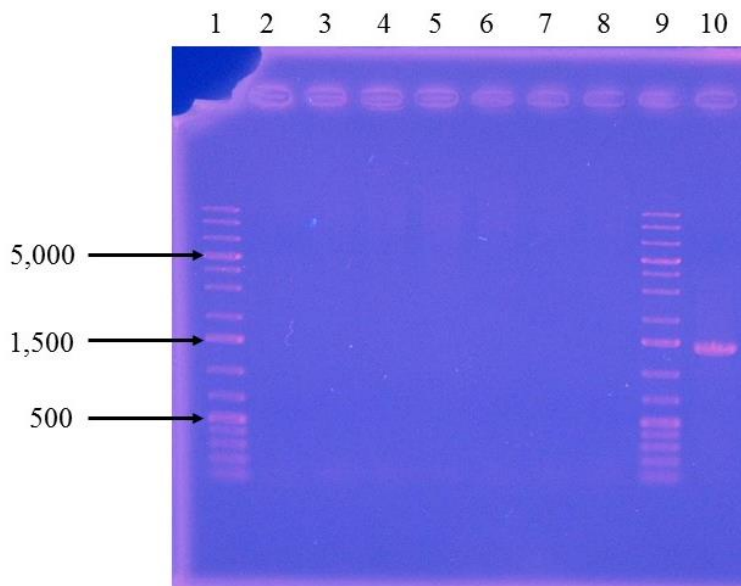
Figure 23. Genomic DNA PCR confirmation amplifying a region spanning from inside the site of deletion to outside of the upstream flanking region. PCR samples were run on 1% agarose gel in comparison to GeneRuler 1kb DNA Ladder. DNA bands were visualized using EtBr and UV light. Lane 1 & 9: GeneRuler 1kb DNA Ladder; lane 2-8: potential LAC-4 ΔHOC transformants; lane 10: LAC-4 wildtype (1,389 bp). See Fig. 16C for primer orientation.
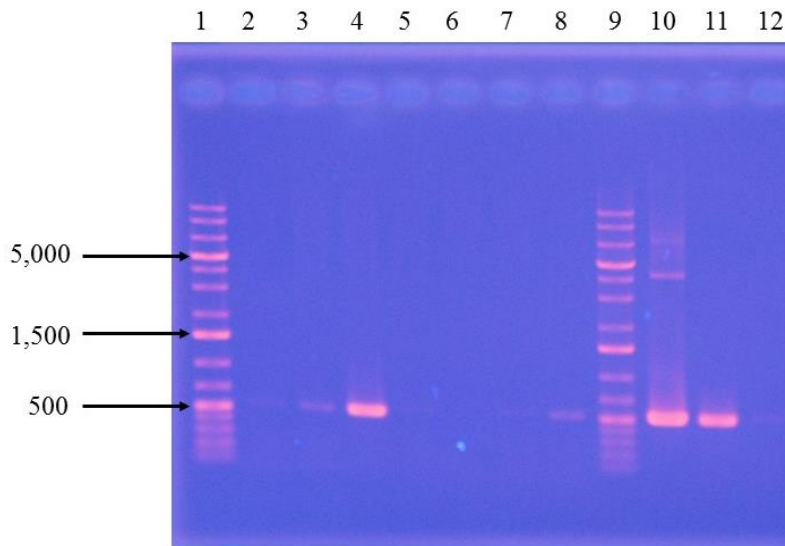
44
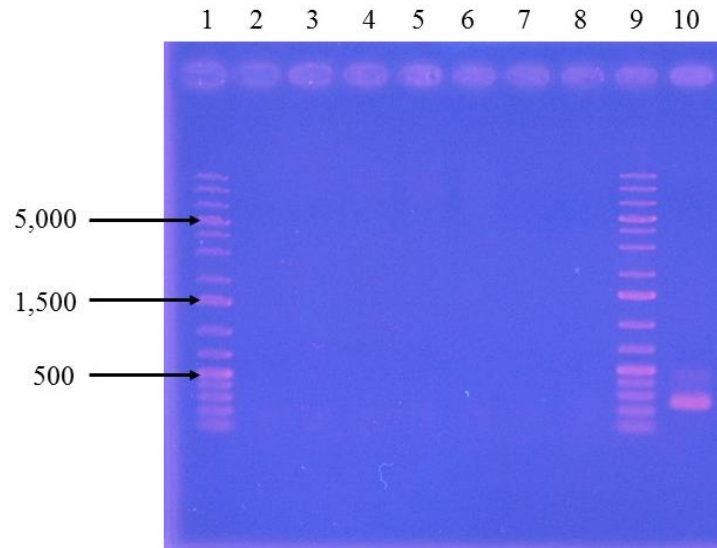
Figure 24. Genomic DNA PCR confirmation for the absence of the *hemO* gene. PCR samples were run on 1% agarose gel in comparison to GeneRuler 1kb DNA Ladder. DNA bands were visualized using EtBr and UV light. Lane 1 & 9: GeneRuler 1kb DNA Ladder; lane 2-8: potential LAC-4 ΔHOC transformants; lane 10: LAC-4 wildtype (240 bp). See Fig. 21B for primer orientation.

Figure 25. Genomic DNA PCR pMo130 backbone confirmation for absence of the *kanR* gene. PCR samples were run on 1% agarose gel in comparison to GeneRuler 1kb DNA Ladder. DNA bands were visualized using EtBr and UV light. Lane 1 & 9: GeneRuler 1kb DNA Ladder; lane 2-8: potential LAC-4 ΔHOC transformants; lane 10: pMo130 (520 bp); lane 11: LAC-4 HOC single-crossover mutant (520 bp); lane 12: LAC-4 wildtype.

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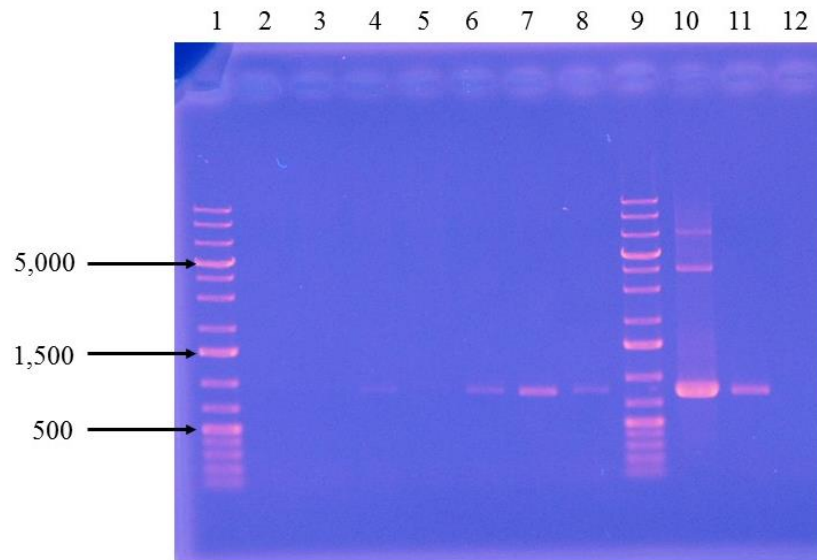
Figure 26. Genomic DNA PCR pMo130 backbone confirmation for absence of the *sacB* gene. PCR samples were run on 1% agarose gel in comparison to GeneRuler 1kb DNA Ladder. DNA bands were visualized using EtBr and UV light. Lane 1 & 9: GeneRuler 1kb DNA Ladder; lane 2-8: potential LAC-4 ΔHOC transformants; lane 10: pMo130 (860 bp); lane 11: LAC-4 HOC single-crossover mutant (860 bp); lane 12: LAC-4 wildtype.
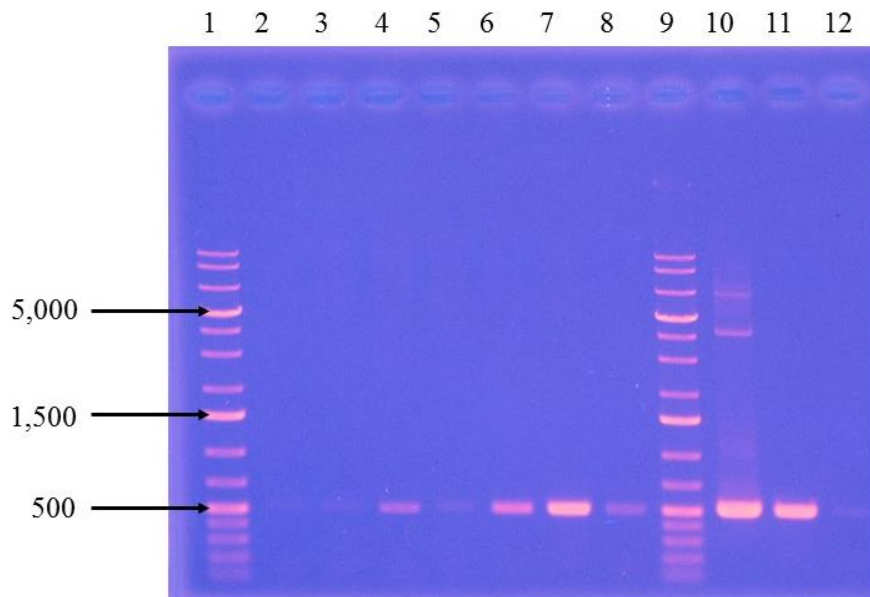

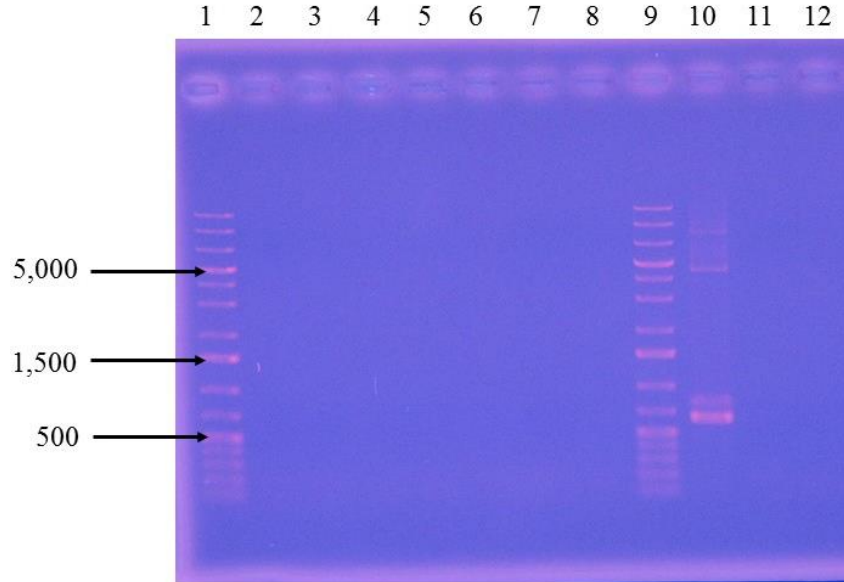*Figure 27*. Genomic DNA PCR pMo130 backbone confirmation for absence of the *xylE* gene. PCR samples were run on 1% agarose gel in comparison to GeneRuler 1kb DNA Ladder. DNA bands were visualized using EtBr and UV light. Lane 1 & 9: GeneRuler 1kb DNA Ladder; lane 2-8: potential LAC-4 ΔHOC transformants; lane 10: pMo130 (501 bp); lane 11: LAC-4 HOC single-crossover mutant (501 bp); lane 12: LAC-4 wildtype.

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Figure 28. Genomic DNA PCR pMo130 backbone confirmation for absence an intergenic region within the pMo130 plasmid. PCR samples were run on 1% agarose gel in comparison to GeneRuler 1kb DNA Ladder. DNA bands were visualized using EtBr and UV light. Lane 1 & 9: GeneRuler 1kb DNA Ladder; lane 2-8: potential LAC-4 ΔHOC transformants; lane 10: pMo130 (630 bp); lane 11: LAC-4 HOC single-crossover mutant (630 bp); lane 12: LAC-4 wildtype.
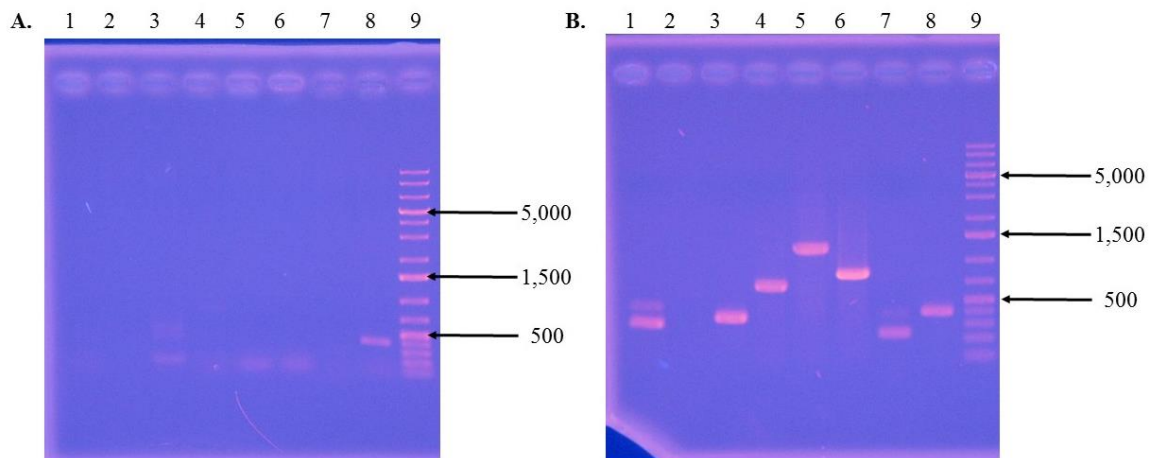
Figure 29. Genomic DNA PCR screen for the individual genes of the *hemO* gene cluster. **A.** The representative knockout, LAC-4 ΔHOC-1; **B**. LAC-4 wildtype. Using the ACICU numbering screen also used in Ou et al., 2015: lane 1: 873 (*VreR*) (342 bp); lane 2: 874 (*VreI)* (854 bp); lane 3: 875 (*hasR*) (389 bp); lane 4: 876 (712 bp); lane 5: 877 (1,314 bp); lane 6: 878 (TonB) (865 bp); lane 7: 879 (*hemO*) (240 bp); lane 8: 880 (412 bp); lane 9: GeneRuler 1kb DNA Ladder. See Fig. 7 for primer orientations.

Sequencing the Junction Site of Deletion

As a final verification, the PCR product from the PE_HOC_CFM_UP & PE_HOC_CFM_DN reaction was purified and prepared for sequencing. Four samples were chosen based on the previous PCR confirmations as the most likely to be clean knockouts that did not retain any portions of the pMo130 backbone or other genetic elements. The four samples were LAC-4 ΔHOC-1, ΔHOC-2, ΔHOC-5 and ΔHOC-7 and were sequenced initially by City of Hope Core Sequencing Lab (Duarte, CA), and subsequently LAC-4 ΔHOC-1 was sequenced again by Retrogen, Inc. (San Diego, CA). Sequence analysis was focused on identifying the bridging region of the flanking regions that would mark the site of deletion. Upstream we looked for a region of 5'-GAGTTTGGCTGATTTGCTTTAA-3' linked up to a downstream region of 5'-TTATGACTGGCATTTTTGCATG-3'. All four samples verified the completed linked sequence containing the fusion of the upstream and downstream bridged regions, 5'-GAGTTTGGCTGATTTGCTTTAAGTTATGACTGGCATTTTTGCATG-3'.

Phenotypic Verification of the *hemO* Gene Cluster Knockout

In order to add confidence to our validation of the ΔHOC mutant, we performed phenotypic tests to compare the knockout strain to the wildtype. The following antibiotic susceptibilities were compared: ampicillin, apramycin, kanamycin, meropenem, tetracycline and ciprofloxacin. Overnight growth was assessed confirming both the cluster knockout and the wildtype shared similar antibiotic resistance and susceptibility profiles (Fig. 30).
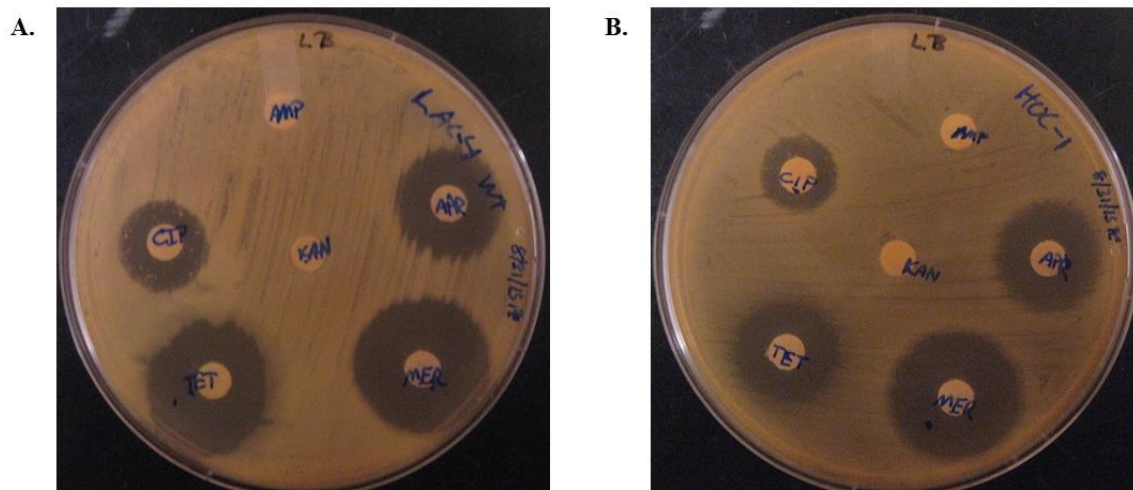
Figure 30. Comparative phenotype assay using disc diffusion and six antibiotics. **A.** presents LAC-4 wildtype; **B.** presents the representative knockout mutant, LAC-4 ΔHOC-1. Plates were thoroughly streaked to produce a bacterial lawn. Diffusion discs were placed and 10 µl of the following antibiotics were added where labeled: ampicillin, apramycin, kanamycin, meropenem, tetracycline and ciprofloxacin.

In Vivo Virulence Assay of the hemO Gene Cluster Knockout

Samples of the validated *A. baumannii* LAC-4 ΔHOC-1 were sent to our collaborator Dr. Wangxue Chen of the National Research Council Canada to be compared with *A. baumannii* LAC-4 wildtype samples previously sent. Additionally, an *A. baumannii* LAC-4 Δ*hemO* single gene knockout that had been previously constructed, had already been validated and was also sent for *in vivo* testing. Results showed that the *hemO* gene cluster mutant had reduced virulence and bacterial burden. Specifically the lung bacterial burden was reduced 1,000 fold compared to wildtype, and blood and spleen bacterial quantification yielded between 100 to 1,000 fold reductions as well (Fig. 31). The single gene *hemO* mutant did not show significant difference compared to wildtype LAC-4.
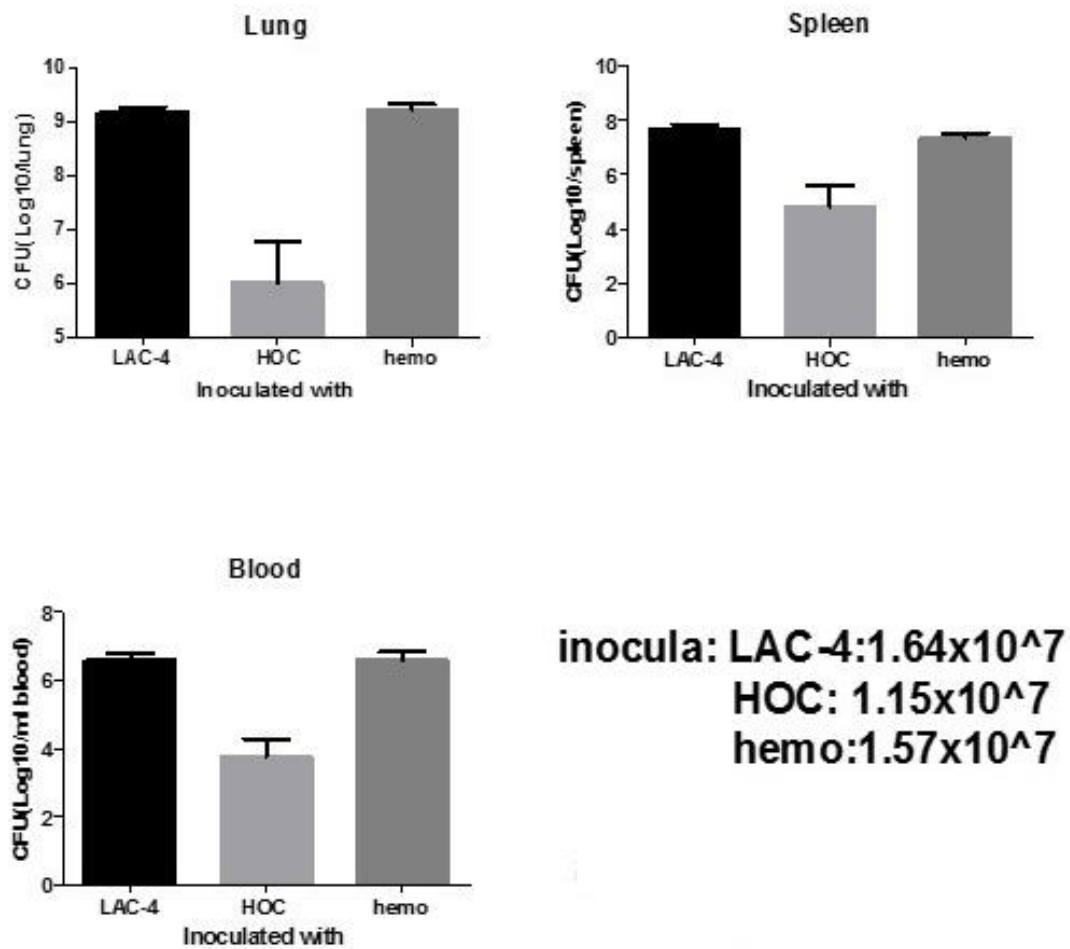
Figure 31. *In vivo* murine study comparing virulence between wildtype LAC-4 and mutant strains. *In vivo* testing completed by our collaborator comparing the bacterial burden in the lungs, spleen and blood amongst wildtype LAC-4, a *hemO* cluster knockout, and a *hemO* gene knockout we provided.

CHAPTER 4

Discussion

The emergence and increasing prevalence of multi-drug resistant and pan-resistant bacterial strains threatens to overtake our ability to treat MDR infections with conventional antibiotics (Bassetti et al., 2015; Boucher et al., 2009). New antimicrobial drug discovery has sharply decreased since the golden age of antibiotic discovery, and in the past thirty years only two new classes of antibiotics were approved for clinical use, yet many researchers and medical professionals have remained confident in their ability to modify existing antibiotics to stay ahead of ever evolving pathogens (Aminov, 2010; Jagusztyn-Krynicka et al., 2008). In response to the growing clinical prevalence of MDR strains, there has been a call to action to bring forth new antibiotics (Boucher et al., 2013). With limited treatment options against emerging MDR strains, it has been important to consider non-conventional treatments such as targeting virulence, an approach that may help change the course of an infection without necessarily killing bacteria, potentially allowing the host immunity along with conventional antibiotics to clear the infection.

While traditional antibiotic therapy relies upon chemical agents that are either inhibitory or lethal to bacteria, targeting virulence factors is more akin to disarming pathogens (Allen et al., 2014). By removing or disabling a pathogen's ability to cause disease, pathogen growth may be halted, the ability to find a growth niche in the host's commensal flora prevented, and in some cases the pathogen may be cleared by host immunity (Clatworthy et al., 2007). Methods of targeting virulence can include inhibiting bacterial toxin function or delivery, targeting virulence expression regulators, preventing

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biofilm formation and bacterial adhesions, as well as hindering specialized bacterial secretory systems (Clatworthy et al., 2007; Rasko et al., 2010). Even if virulence cannot be completely controlled or reduced, it has been hypothesized that virulence targeting compounds could be used synergistically with conventional antibiotics, thereby reducing therapeutic doses needed of the said antibiotics (Rasko et al., 2010).

Recently there has been progress in the development of antivirulence inhibitors, with many in some phase of development or animal testing (Rasko et al., 2010). Most of the inhibitors in development exert their actions by inhibiting bacterial toxins, adhesions, quorum sensing and secretion systems (Allen et al., 2014; Rasko et al., 2010). Of the significant antivirulence inhibitors being developed, none have addressed virulence factors that contribute to a bacterium's ability to enter the bloodstream and cause a successful bacteremia. This deficiency may in part be due to a poor understanding of the mechanisms by which bacterium systemically disseminates from a local infection, and even more so for microorganisms that have not been studied in detail like *A. baumannii* (de Léséleuc et al., 2014). While such mechanisms are yet to be elucidated, advances in the understanding of iron metabolism have suggested its role in virulence as an obvious target in limiting systemic infections (de Léséleuc et al., 2012). Interestingly, several studies have shown some success in using iron chelators in the treatment of bacterial and fungal infections (Fernandes et al., 2010; Ibrahim et al., 2011). This is of little surprise because a common characteristic of most virulent bacteria is acquiring iron from the host in other-wise iron poor locations (de Léséleuc et al., 2012).

In 2012, de Léséleuc et al., showed that gallium nitrate, the salt of a non-oxidizable transition metal that resembles ferric iron (Fe^{3+}), could inhibit growth of *A.*

baumannii and reduce overall lung bacterial burden in mice. While not a chelating agent, gallium nitrate is theorized to work synergistically with transferrin in the blood stream (de Léséleuc et al., 2014). This likely works due to most free iron in the blood being sequestered by transferrin, meanwhile gallium is thought to enter bacterial cells through their iron uptake mechanisms, disrupting or interfering with iron metabolism, DNA synthesis and protein synthesis (Bernstein, 1998). Gallium nitrate thus was proposed as a potentially simple antivirulence antimicrobial that would work as a competitive inhibitor of *A. baummannii*'s siderophores (de Léséleuc et al., 2012; de Léséleuc et al., 2014). In 2014, de Léséleuc et al., showed that the hypervirulent *A. baumannii* strain, LAC-4, was tolerant to gallium nitrate, and its growth was not inhibited and LAC-4 continued with its extrapulmonary dissemination. LAC-4 was later discovered to possess a heme oxygenase enzyme and was apparently acquiring its needed iron from heme (de Léséleuc et al., 2014). This increased heme utilization was then challenged with gallium protoporphyrin IX, a heme analog that appears to block heme acquisition mechanisms, and was thus able to attenuate the hypervirulent strain (de Léséleuc et al., 2014). Overall, LAC-4's ability to overcome the gallium nitrate treatment, a therapy that would have halted less virulent strains of *A. baumannii,* demonstrates that similar to MDR strains, bacterium resistant to antivirulence therapies will likely emerge. This further highlights the need to clearly characterize and identify the mechanisms of action for antivirulence therapeutics as well as conventional antibiotics. Genomics and molecular genetic techniques are prime for this, and can be used to characterize and confirm targets, as well as elucidate new antimicrobial strategies (Pucci et al., 2006).

In this study we hypothesized that *A. baumannii* LAC-4's hypervirulence was associated with its possession of a second heme utilization gene cluster, the *hemO* gene cluster. While a general ability of *A. baumannii* strains to utilize heme is likely due to the ubiquitous presence of a heme utilization cluster in *A. baumannii* (ABLAC_24350 – ABLAC_24460 in LAC-4), it does not explain LAC-4's unusually high heme consumption nor does it appear to contribute to LAC-4's hypervirulence. We and our collaborators had determined that LAC-4 was highly efficient at metabolizing heme, so much so that when heme utilization was blocked, LAC-4 grew more slowly than the *A. baumannii* laboratory strain ATCC 17978 (de Léséleuc et al., 2014). ATCC 17978 does not contain the *hemO* gene cluster that was present in LAC-4, and contains only the general heme utilization cluster seen in all *A. baumannii*. Thus it has been hypothesized then that LAC-4's *hemO* gene cluster, and specifically its heme oxygenase gene (*hemO*), were responsible for LAC-4's high heme consumption and ability to efficiently disseminate from the lungs (de Léséleuc et al., 2014).

To test our hypothesis, we constructed a *hemO* gene cluster KO to assess this cluster's role in LAC-4's hypervirulence. Additionally, in order to address the issue of having few molecular genetics tools in MDR *A. baumannii* strains, we set about making this mutant unmarked in addition to optimizing existing counter-selection techniques allowing for the reliable production of mutants in *A. baumannii* via homologous recombination. The *hemO* gene cluster KO cassette was constructed using overlap extension PCR technology and the mutant was made unmarked by linking the apramycin resistance gene outside of the cassette (Oh et al., 2015). Placement of the antibiotic resistance selection marker outside of the cassette allowed for its subsequent genomic

removal during the second-crossover event, thereby allowing the marker to be recycled when it is necessary to construct multiple gene deletions in a single strain. After successful cassette construction and transformation into our intermediate host, *E. coli* DH5α, plasmid was grown and extracted and verified to contain the entire KO cassette. We then electroporated the suicide plasmid containing KO cassette into *A. baumannii* LAC-4. Once plated on selective media, only LAC-4 colonies that had integrated the suicide vector during a single-crossover event, co-integrants, grew on the apramycin containing media.

Our use of the *xylE* reporter gene proved useful as a quick method of distinguishing between successful co-integrants and false positives during the single-crossover event, while additionally serving as a means to determine which colonies had lost the suicide vector backbone via homologous recombination after the second-crossover event. Co-integrant colonies that contained the *xylE* gene produced catechol-2,3-dioxygenase, an enzyme that acts upon catechol-like substrates to produce yellow product identical or similar to 2-hydroxymunoic semialdehyde which is revealed as a bright yellow pigment in the medium (Lee et al., 1996). Using this reporter gene in conjunction with sucrose based counter-selection allowed us to optimize our knockout method, producing greater consistency and repeatability. Obtaining ideal conditions for counter-selection was difficult, because it appears that LAC-4 is not as sensitive to the levans or the amount of levans produced by the *sacB* encoded levansucrase. Passaging in sucrose containing medium for three days did show significant stress on cell growth, but ultimately upon plating, the levans produced in the continued presence of sucrose appeared to not be very inhibitory. Co-integrate colonies continued to grow alongside

wildtype revertants and KO mutants on sucrose containing medium in spite of *sacB*'s putative counter-selection capability. The use of *xylE* allowed us to avoid a massive colony PCR screening by narrowing our selection of colonies to those that did not express our reporter gene. This screening process was enormously important in reducing the time-consuming workload in identifying successful KOs. On average, we counted approximately 49 colonies per plate, of which only 4% were white in color. Of the white colonies, only 34.5% were verified to be our ΔHOC mutant, with the remaining being identified as wildtype revertants. Our method of making use of a reporter gene during counter-selection allowed us to avoid screening 96% of the colonies, and facilitated quicker identification of the 1.4% of colonies on the counter-selection medium that were in fact our mutant.

To determine the *hemO* clusters impact on virulence, our collaborators performed an *in vivo* virulence assay using an established mouse model of bacterial pneumonia on the unmarked ΔHOC mutant we had generated as well as another *hemO* single gene mutant we had previously generated (Harris et al., 2013). The results clearly indicated that the *hemO* gene cluster significantly contributed to LAC-4's hypervirulence, though there are likely other factors contributing as well. While lung bacterial burden was dramatically reduced by nearly a thousand fold, and bacterial load in the blood and spleen was reduced just over a hundred fold. Furthermore, the mice experienced a mild bacteremia in comparison to the wildtype LAC-4 (unpublished data). These results indicate that LAC-4's heme utilization is critical to its ability to grow in high numbers in the lungs and to disseminate to other organs, though the absence of the *hemO* gene cluster does not prevent a bacteremia from occurring. Once in the circulatory system, LAC-4

ΔHOC does proliferate, but a hundred fold less in comparison to wild-type, resulting in the mouse experiencing an attenuated infection in comparison. This suggests that LAC-4 ΔHOC is still able to acquire sufficient heme or iron from other mechanisms to maintain a modest infection regardless of losing the *hemO* gene cluster. This is not at all unreasonable given that LAC-4 does contain another common heme utilization cluster and several iron siderophore gene clusters (Ou et al., 2015). It also raises questions about how heme utilization occurs within the lungs, as the greatest bacterial burden decrease in comparison to wild-type was observed there.

LAC-4's ability to rapidly metabolize heme had been hypothesized to be linked to the heme oxygenase gene encoded within the *hemeO* gene cluster (de Léséleuc et al., 2014). However, the *in vivo* results of LAC-4 Δ*hemO* mutant indicate no reduction in bacterial burden during infection. This result was surprising, as it indicates that the *hemO* gene is not responsible for LAC-4's hypervirulence. Instead, it suggests that one or more other genes in the *hemO* gene cluster may be responsible for the sharp reduction in the growth seen with LAC-4 ΔHOC. Once again, given LAC-4's possession of another heme utilization cluster ubiquitous to all *A. baumannii*, it is reasonable to presume that LAC-4 still retained other mechanisms to obtain ferrous iron from heme. This results in a paradigm shift for possible explanations for the *hemO* gene cluster's role in hypervirulence, and suggests that it is the highly efficient heme uptake system in the gene cluster that is most critical for virulence, rather than the actual ability to oxidatively cleave heme.

It is likely that the deletion of one gene or several genes involved in the assembly of the TonB dependent receptor would retard LAC-4's ability to import extracellular

heme, thereby reducing LAC-4's virulence as seen in absence of the entire cluster. Furthermore, prior studies of homologous gene clusters to the *hemO* gene cluster in other pathogenic species have identified some of these genes as virulence factors (Llamas et al., 2009). Particularly, the ECF sigma factor and its anti-sigma factor encoded by *vreR* and *vreI* respsectively, along with the *hasR* enconded outer-membrane receptor, have been characterized in regulating virulence and have even been found in some species to cohabit genetic islands with antibiotic resistance genes (Benevides-Matos et al., 2010; Llamas et al., 2009; Luck et al., 2001). Further study on these individual genes is warranted to determine which gene or genes is the putative contributor to hypervirulence of LAC-4, as well to further elucidate the functions and roles of these specific genes in heme acquisition.

While these results did not provide a definitive mechanistic explanation for the *hemO* gene cluster's involvement in LAC-4's hypervirulence, they did confirm its role as a major contributor to that virulence. The LAC-4 ΔHOC mutant showed significant reduction in virulence, and set the ground work for additional work in our lab to narrow down and identify the contributing genes for LAC-4's hypervirulence. This work not only helps elucidate the roles of the *hemO* gene cluster in LAC-4, but also provides insight into other strains, often nosocomial strains, of *A. baumanii* and other species which possess such homologous gene clusters. Additionally, the optimization of gene deletion methods using both a *sacB* counter-selection and a *xylE* reporter gene will facilitate molecular genetics studies in MDR *A. baumannii* strains, as well as provide a framework to develop an even more robust unmarked genome editing system. The lack of genetics tools to work efficiently on Gram-negative MDR strains, as well as the need for

new novel treatment options, is of the utmost value due to the ever increasing prevalence of MDR strains. And although our laboratory's work on the involvement of heme acquisition in virulence, as well as our development of genetics tools for MDR *A. baumannii*, are far from accomplished, our contributions have set the stage for future experimentation and have helped gain greater understanding of the mechanisms of virulence in highly pathogenic multi-drug resistant bacterium.

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